IN THE UNITED STATES PATENT AND TRADEMARK OFFICE (USPTO)	
Application Serial Number	TBD
Confirmation Number	TBD
Filing Date	Herein
Title of Application	Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension
First Named Inventor	James S. Baldassarre
Assignee	Ikaria, Inc.
Group Art Unit	TBD
Examiner	TBD
Attorney Docket Number	I001-0002USC1

Pre-Examination Search Document

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This pre-examination search statement is provided in support of the Petition for Accelerated Examination filed herewith.

A pre-examination search was conducted involving U.S. patents and patent application publications, foreign patent documents and non-patent literature as indicated below. The results of the search are provided on an Information Disclosure Statement filed concurrently herewith.

The search primarily includes the following aspects:

- The method of reducing adverse events in patients in need of treating with nitric oxide - excluding patients with pre-existing left ventricular dysfunction.
- The patients have a pulmonary capillary wedge pressure greater than 20mm Hg.
- Patients with left ventricular dysfunction have conditions like systolic or diastolic dysfunction, hypertensive, viral, iodopathic cardiomyopathy, autoimmune disease related cariomyopathy, structural heart disease, idiopathic pulmonary arterial hypertension, pulmonary hypertension cardiomyopathy.

- The patient's population are children and adults.
- Adverse events are pulmonary edema, hypotension, cardiac arrest, ECG changes, hypoxemia, hypoxia and bradycardia.
- The patient in need of nitric oxide inhalation has PCWP<=15mg, PVRI>3micro.sq.meters.
- Left ventricular afterload is minimized by administering a pharmaceutical dosage form comprising nitroglycerin and calcium channel blocker to the patient, using an inter-aortic balloon pump.

8 (A) Pre-examination Search

Details of US Patent Classification Codes used

http://www.uspto.gov/go/classification/

128-Surgery

128/200.14 – Liquid Medicament Atomizer or Sprayer

128/200-24 – Respiratory Method or Device

128/203.15 – Particular treating agent carried by breathes gas

128/203.12 – Means for mixing treating agent with respiratory gas

558- Organic Compounds

558/486 – Glyceryl trinitrate per se (i.e., trinitroglycerin)

423 – Chemistry or Inorganic Compounds

423/405 – Nitric Oxide (NO)

600 – Surgery

600/481 – Cardiovascular

600/513 – Detecting heartbeat electric signal and diverse cardiovascular characteristic

Details of IPC-8 Codes used

http://www.wipo.int/classifications/ipc/ipc8/?lang=en

A61K – Preparations for Medical, Dental, or Toilet Purposes

A61K 33/00 – Medicinal preparations containing inorganic active ingredients

A61K 33/08 – Oxides; Hydroxides

A61P – Specific Therapeutic Activity of Chemical Compounds or Medicinal Preparations

A61P 9/00 – Drugs for disorders of the cardiovascular system

A61P 9/04 – Inotropic agents, i.e. stimulants of cardiac contraction; drugs for heart failure

A61P 9/08 – Vasodilators for multiple indications

A61P 43/00 – Drugs for specific purposes

C01B – Non-Metallic Elements; Compounds Thereof

C01B 21/24 – Nitric oxide (NO)

Dates Conducted: May 10, 2010 and May 17, 2010

Database Searches

Database Service: Legal Advantage

Data Searched: All patents and Non-patent literature

Database Used: MicroPatent, USPTO, European Patent Office/Espacenet, WIPRO, JPO, Google, Springerlink, Wiley Interscience, ScienceDirect, Scirus, Journal of Medicinal Chemistry, ACS Publications, and, Journal of American Academy of Pediatrics.

Search Logic

Search No.	Concept	Keywords
1	Nitric oxide	Nitric oxide, nitrogen monoxide, nitrogen oxide, iNO, NO
2	Inhale	Inhale, breath, gasp
3	Reduce	Reduce, minimize, prevent, avoid, exclude, reject, except, omit
4	Adverse event	Adverse/undesirable/unfavorable/unfavorable event/effect/consequence/indication, side effect, toxicity, toxin
5	Identify	Identify, select, choose, opt, pick, screen, find, segregate, separate, distinguish, take out
6	Left ventricular dysfunction	Left ventricular dysfunction, LVD, diastolic/systolic dysfunction, cardiomyopathy, heart disease
7	Pulmonary Capillary wedge pressure	Pulmonary Capillary wedge pressure, PCWP
8	Respiratory failure	Respiratory failure, Pulmonary edema, hypotension or cardiac arrest, heart failure, heart attack, electrocardiogram/ECG change, hypoxia, hypoxemia, bradycardia

8(B) Search Directed to the Invention

The pre-examination search was directed to the claimed invention, encompassing all the features of the claims and giving the claims their broadest reasonable interpretation.

8(C) Search Directed to the Disclosure

No disclosed features that are unclaimed at this time are currently seen as features that may be claimed later.

8(D) Search Report from a Foreign Patent Office

Search reports from Australia, Japan, and the EPO are attached herewith.

8(E) Statement of Good Faith

All statements above in support of the petition to make special are based on a good faith belief that the search was conducted in compliance with the requirements of this rule.

Respectfully Submitted,

Christopher P. Rogers, Reg. No. 36,334

Date: 21 June 2010

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15 March 2010

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Your Ref: 28686IKA/AMM:Is

Examiner's first report on patent application no. 2009202685
by Ikaria Holdings, Inc.

Last proposed amendment no.

Dear Madam/Sir,

I am replying to the request for normal examination. I have examined the application and I believe that there are lawful grounds of objection to the application. These grounds of objection are:

1. The invention defined in claims 1-30 does not involve an inventive step when compared to the disclosure of each of the following prior art documents*:

   D1: LOH, E. *et al.* "Cardiovascular Effects of Inhaled Nitric Oxide in Patients with Left Ventricular Dysfunction". CIRCULATION, 1994, vol.90: 2780-2785.
   D2: CUJEC, B. *et al.* "Inhaled Nitric Oxide Reduction in Systolic Pulmonary Artery Pressure is Less in Patients with Decreased Left Ventricular Ejection Fraction". CANADIAN JOURNAL OF CARDIOLOGY, 1997, vol.13(9): 816-824.
   D3: ROSALES, A *et al.* "Adverse Hemodynamic Effects Observed with Inhaled Nitric Oxide After Surgical Repair of Total Anomalous Pulmonary Venous Return". PEDIATRIC CARDIOLOGY, 1999, vol.20: 224-226.
   D4: BOCCHI, E. *et al.* "Inhaled Nitric Oxide Leading to Pulmonary Edema in Stable Severe Heart Failure". THE AMERICAN JOURNAL OF CARDIOLOGY, 1994, vol.74: 70-71.
   D5: ARGENZIANO, M. *et al.* "Inhaled Nitric Oxide is not a Myocardial Depressant in a Porcine Model of Heart Failure". THE JOURNAL OF THORACIC AND CARDIOVASCULAR SURGERY, 1998, vol.115: 700-704.

   The problem addressed by the current application is reducing adverse events or serious adverse events associated with inhaled nitric oxide in patients who have pre-existing left ventricular dysfunction.

   The cited art is directed to a problem similar to the applicant's problem, and in searching the problem a person skilled in the art could reasonably be expected to have found, and to have ascertained, understood, and regarded, this prior art as relevant.

   D1 investigated the use of inhalation of the pulmonary vasodilator, nitric oxide (NO), in patients with heart failure due to left ventricular dysfunction (LVD). The cause of heart failure in half the patients was ischemic cardiomyopathy and in the other half it was caused by idiopathic dilated cardiomyopathy (see abstract and Methods: Study Population). Following

administration of NO via a face masks patients showed an increase in the mean pulmonary artery wedge pressure associated with decreases in cardiac index and stroke volume index (see Results). It is suggested that selective pulmonary vasodilation is not desirable in patients with left ventricular failure (see page 2784, last paragraph).

D2 discloses that there have been reports that a decrease in pulmonary vascular resistance following iNO inhalation occurs in patients with LVD as a result of an increase in pulmonary capillary wedge pressure. D2 further investigated the effects of iNO in a group of patients with a broad range of left ventricular function in a randomized manner (see page 817, left col.). Some of the patients received oxygen in addition to NO (see page 818, Study protocol). Three patients with depressed left ventricular ejection fraction (LVEF) presented with pulmonary oedema after administration of nitric oxide (see page 821, left col. 1st paragraph and page 822, right col., lines 4-6). Other adverse events to occur in patiens with depressed LVEF were an increase in pulmonary wedge pressure and decreased pulmonary vascular resistance (the latter patients were also cardiomyopathy patients) (see page 821, right col.). There is a clear suggestion that the use of nitric oxide is limited in patients with pre-existing LVD (see CONCLUSIONS).

D3 discloses a case report of a one month old patient who underwent corrective surgery with pulmonary vein confluence to left atrial anastomosis (see abstract). The patient was treated with NO therapy following development of sudden onset systemic-level pulmonary pressure with concomitant systemic hypotension. However, favourable changes were followed by "rebound" pulmonary hypertension that occurred with concomitant systemic hypotension and central venous pressure. Therapy with NO was discontinued based on the rationale that this episode of pulmonary hypertension may have been caused by left atrial hypertension secondary to a sudden increase in pulmonary blood flow into a noncompliant left atrium and ventricle (see page 225, 4th and 5th paragraphs). As a result, D3 states that NO therapy can be detrimental in patients with LVD and/or cardiomyopathy as these patients may develop pulmonary oedema (see abstract and page 226, left col., last paragraph).

D4 pertains to a study in which patients with refractory heart failure and severe pulmonary hypertension having impaired LVEF and severe and diffuse systolic dysfunction were administered NO via inhalation. Following NO therapy patients presented with an increase in pulmonary wedge pressure and developed pulmonary oedema (see whole document).

D5 discloses that there have been reports of increases in left ventricular end-diastolic pressure and episodes of pulmonary oedema during the clinical use of inhaled nitric oxide (iNO) in patients with pre-existing LVD (see abstract and the introduction).

Each of D1-D5 differs from the instant specification in that they do not specifically disclose excluding patients with LVD from iNO treatment nor the steps of informing a medical provider that excluding patients with LVD from iNO treatment reduces adverse events. However, each of D1-D5 discloses that adverse events occur in patients with pre-existing LVD following administration of iNO and they clearly suggest that precautions should be taken when administering iNO.

Therefore the person skilled in the art would directly and without difficulty, by routine steps, arrive at a solution which is the same as the claimed solution, and therefore the claimed invention lacks an inventive step.

* As found during a national phase search

NOTE: There is a current postponement of acceptance in place. If you overcome all other objections before the expiration of that postponement, the Commissioner will only accept the application at that time if you have filed a clear and unambiguous statement requesting the withdrawal of that postponement. Otherwise, a further adverse report will be issued.

You have 21 months from the date of this report to overcome all my objection(s) otherwise your application will lapse.

You will need to pay a monthly fee for any response you file after 12 months from the date of the first report.

You will also need to pay any annual continuation fees that apply. These will normally be first due five years from the filing date. Please note however that earlier commencement dates apply for divisional applications.

Information about fees may be obtained by phoning 1300 651 010.

Yours faithfully,

EDWINA VANDINE
Patent Examination A
A1 - PBR Plants & Biotechnology
Phone: (02) 6225 6113

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GRANDE BRETAGNE

J. A. KEMP & Co
RECD 10 MAY 2010
Action by .....

Date: 10.05.10

Reference	Application No./Patent No.
N.108660-TJD	09251949.5 - 2123

Applicant/Proprietor: Ikaria Holdings, Inc.

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the European search report (R. 61 EPC) or the partial European search report/ declaration of no search (R. 63 EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☑ 1 additional set(s) of copies of such documents is (are) enclosed as well.

The following have been approved:

☑ Abstract ☑ Title

☐ The Abstract was modified and the definitive text is attached to this communication.

The following figure(s) will be published together with the abstract:

Refund of the search fee

If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

Europäisches Patentamt · European Patent Office · Office européen des brevets

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 09 25 1949

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	LOH EVAN ET AL: "Cardiovascular Effects of Inhaled Nitric Oxide in patients With Left Ventricular Dysfunction" CIRCULATION, vol. 90, no. 6, 1994, pages 2780-2785, XP002577161 ISSN: 0009-7322 * the whole document *	1-9	INV. A61K33/00 A61P9/08 A61P9/12
X,D	SEMIGRAN MARC J ET AL: "Hemodynamic effects of inhaled nitric oxide in heart failure" JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY, vol. 24, no. 4, 1994, pages 982-988, XP009131903 ISSN: 0735-1097 * the whole document *	1-9	
X,D	HAYWARD C S ET AL: "Inhaled nitric oxide in cardiac failure: Vascular versus ventricular effects" JOURNAL OF CARDIOVASCULAR PHARMACOLOGY, vol. 27, no. 1, 1996, pages 80-85, XP009131904 ISSN: 0160-2446 * the whole document *	1-9	TECHNICAL FIELDS SEARCHED (IPC) A61K
X	OVODOV ET AL: "Nitric oxide: Clinical applications" SEMINARS IN ANESTHESIA, SAUNDERS, CO, NEW YORK, NY, US LNKD- DOI:10.1053/SA.2000.6785, vol. 19, no. 2, 1 June 2000 (2000-06-01), pages 88-97, XP005426335 ISSN: 0277-0326 * page 90, column 1 * * page 93, column 2 - page 94 *	1-9	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	13 April 2010	Albrecht, Silke

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 09 25 1949

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	HENRICHSEN ET AL: "Inhaled nitric oxide can cause severe systemic hypotension" JOURNAL OF PEDIATRICS, MOSBY-YEAR BOOK, ST. LOUIS, MO, US LNKD-DOI:10.1016/S0022-3476(96)70230-5, vol. 129, no. 1, 1 July 1996 (1996-07-01), page 183, XP022199226 ISSN: 0022-3476 * the whole document *	1-9	
X	ADATIA ET AL: "Inhaled nitric oxide and hemodynamic evaluation of patients with pulmonary hypertension before transplantation" JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY, ELSEVIER, NEW YORK, NY, US LNKD- DOI:10.1016/0735-1097(95)00048-9, vol. 25, no. 7, 1 June 1995 (1995-06-01), pages 1656-1664, XP005857183 ISSN: 0735-1097 * page 1663, column 1 *	1-9	TECHNICAL FIELDS SEARCHED (IPC)
X	CUJEC BIBIANA ET AL: "Inhaled nitric oxide reduction in systolic pulmonary artery pressure is less in patients with decreased left ventricular ejection fraction" CANADIAN JOURNAL OF CARDIOLOGY, vol. 13, no. 9, 1997, pages 816-824, XP002577162 ISSN: 0828-282X * the whole document *	1-9	

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	13 April 2010	Albrecht, Silke

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 09 25 1949

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	FINDLAY G P: "Paradoxical haemodynamic response to inhaled nitric oxide" INTERNATIONAL JOURNAL OF INTENSIVE CARE 1998 GB, vol. 5, no. 4, 1998, pages 134-139, XP001536771 ISSN: 1350-2794 * the whole document *	1-9	
X,D	BOCCHI E A ET AL: "Inhaled nitric oxide leading to pulmonary edema in stable severe heart failure" AMERICAN JOURNAL OF CARDIOLOGY, CAHNERS PUBLISHING CO., NEWTON, MA, US LNKD-DOI:10.1016/0002-9149(94)90496-0, vol. 74, no. 1, 1 July 1994 (1994-07-01), pages 70-72, XP023278686 ISSN: 0002-9149 [retrieved on 1994-07-01] * the whole document *	1-9	

TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	13 April 2010	Albrecht, Silke

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

(Translation of Official Action)

NOTIFICATION OF REASON FOR REJECTION

Mailed: February 23, 2010

Japanese Patent Application No. 2009-157623

Applicant: IKARIA HOLDINGS, INC.

The present application should be rejected for the following reason(s). If the applicant has any argument against the reason(s), an Argument must be filed within three months of the mailing date of this Official Action.

REASON 1

The present invention as claimed in the following claim(s) is unpatentable under Article 29, paragraph 1, sub-paragraph 3 of the Japanese Patent Law as being anticipated by the following publication(s) distributed in Japan or elsewhere or as being identical with an invention made available to the public through electric telecommunications prior to the filing of the present application.

REASON 2

The present invention as claimed in the following claim(s) is unpatentable under Article 29, paragraph 2 of the Japanese Patent Law since the invention could have been easily made by those skilled in the art to which it pertains on the basis of the invention(s) described in the following publication(s) distributed in Japan or elsewhere or an invention/inventions made available to the public through electric telecommunications prior to the filing of the present application.

NOTE:

Citation 1: Inglessis, I. *et al.*, Journal of the American College of Cardiology, 2004, Vol. 44, No. 4, pp. 793-798

Citation 2: Loh, E. *et al.*, Circulation, 1994, 90, pp. 2780-2785

Citation 3: Steinhorn, R.H. *et al.*, Pulmonary Hypertension, Persistent-Newborn, emedicine, updated Apr. 19, 2007 [http://emedicine.medscape.com/article/898437-overview]

Citation 4: BOCCHI, E.A. *et al.*, The American Journal of Cardiology, 1994, Vol. 74, pp. 70-72

A.

Reasons 1 and 2/ Claims 1 to 14/ Citation 1

Citation 1 discloses that inhaled nitric oxide is known as a selective pulmonary vasodilator (Abstract), and that inhaled nitric oxide, when administered to patients with right ventricular myocardial infarction and cardiogenic shock, reduced the pulmonary arterial pressure (Abstract). Citation 1 also discloses that the inhalation of nitric oxide is known to decrease pulmonary vascular tone in adults and children with pulmonary hypertension (page 793, right column, lines 11 to 6 from the bottom), and that nitric oxide is delivered by means of a ventilator or is mixed with oxygen (page 795, left column, "NO administration"). Especially, Table 2 presents hemodynamic parameters of target patients at the time of study enrollment, indicating that most of the patients have a pulmonary capillary wedge pressure (PCWP) of less than 20 mmHg.

In light of the present specification (paragraph [0013]), the patients of Citation 1 having a PCWP of less than 20 mmHg are not deemed to have pre-existing left ventricular dysfunction (LVD).

Thus, the present invention as claimed in claims 1 to 14 is indistinguishable from the invention disclosed in Citation 1.

(The present invention and the invention disclosed in Citation 1 are identical in active ingredient and target patients, and thus are deemed to necessarily provide the same functions/effects.)

B.

Reason 2/ Claims 1 to 14/ Citations 1 to 4

Inhaled nitric oxide is well known as a selective pulmonary vasodilator, as disclosed in Citation 1.

On the other hand, Citation 2 (for example, Abstract) discloses that inhaled nitric oxide, when administered to patients with left ventricular dysfunction, may cause a decrease in pulmonary vascular resistance associated with an increase in left ventricular filling pressure, leading to the risk of the occurrence of adverse events.

Citation 3 (for example, see Abstract and "Treatment with iNO") discloses that, although inhaled nitric oxide is used for the treatment of pulmonary hypertension of newborns, patients suffering from congenital cardiac disease characterized by left

ventricular outflow tract obstruction and severe left ventricular dysfunction have a contraindication to the treatment with inhaled nitric oxide.

Citation 4 (page 71, left column, lines 13 to 15) discloses that inhaled nitric oxide, when administered to patients with severe heart disease, may cause pulmonary edema.

In view of the above, it would have been obvious to those skilled in the art to exclude patients with pre-existing left ventricular dysfunction from patients to be treated with a selective pulmonary vasodilator, in order to avoid the occurrence of adverse events, based on Citations 1 to 4.

Further, the present invention as claimed in claims 1 to 14 is not deemed to provide particularly remarkable advantages, in view of Citations 1 to 4.

REASON 3
The present application should be rejected on the grounds that the recitation of the claim(s) fails to meet the requirement of Article 36, paragraph 6, sub-paragraph 2 of the Japanese Patent Law in the following respect(s).

NOTES:

(1) The abbreviations "PAPm," "PCWP" and "PVRI" are unclear in meaning.

(2) The term "near" renders the scope of the claimed invention unclear, and thus is inappropriate as an expression for use in the claims.

Background Art Information*

Field of Search: IPC A61K33/00

*The information provided herein constitutes no reason for rejection.

拒絶理由通知書

特許出願の番号	特願２００９－１５７６２３
起案日	平成２２年　２月　９日
特許庁審査官	辰己　雅夫　４４９８　４Ｃ００
特許出願人代理人	吉武　賢次（外　３名）　様
適用条文	第２９条第１項、第２９条第２項、第３６条

この出願は、次の理由によって拒絶をすべきものです。これについて意見がありましたら、この通知書の発送の日から３か月以内に意見書を提出してください。

理　由

１．この出願の下記の請求項に係る発明は、その出願前に日本国内又は外国において、頒布された下記の刊行物に記載された発明又は電気通信回線を通じて公衆に利用可能となった発明であるから、特許法第２９条第１項第３号に該当し、特許を受けることができない。

２．この出願の下記の請求項に係る発明は、その出願前に日本国内又は外国において頒布された下記の刊行物に記載された発明又は電気通信回線を通じて公衆に利用可能となった発明に基いて、その出願前にその発明の属する技術の分野における通常の知識を有する者が容易に発明をすることができたものであるから、特許法第２９条第２項の規定により特許を受けることができない。

３．この出願は、特許請求の範囲の記載が下記の点で、特許法第３６条第６項第２号に規定する要件を満たしていない。

記　　（引用文献等については引用文献等一覧参照）

Ａ．
・理由　１，２
・請求項　１－１４
・引用文献等　１
・備考：
引用文献１には、吸入用一酸化窒素は選択的肺血管拡張剤として知られていること（Abstract）、右心室心筋梗塞および心臓ショックを有する患者に吸入用一

酸化窒素を投与したところ、肺動脈圧が減少したこと（Abstract）が記載されている。同文献にはまた、一酸化窒素の吸入は、成人や小児の肺高血圧患者の肺血管緊張を減少させることが知られていること（p.793 右欄下から11行-下から6行）、ベンチレーターを使用して送達することや酸素と混合すること（p.795 左欄"NO administration"）についても記載されており、特に、Table2には、対照患者の試験登録時の血行動態パラメーターが記載され、多くの患者の肺毛細血管楔入圧（ＰＣＷＰ）が２０ｍｍＨｇ未満であることが示されている。

ここで、本願明細書【００１３】の記載からみて、引用文献１のＰＣＷＰが２０ｍｍＨｇ未満の患者は、先在性左心室機能障害（ＬＶＤ）を有していないものと認められる。

してみると、請求項１－１４に係る発明は引用文献１に記載された発明と区別することができない。

（本願発明と引用文献１記載の発明は、有効成分と対象患者が同一であるから、当然に同様の作用効果を奏するものといえる。）

Ｂ.

・理由　２

・請求項　１－１４

・引用文献等　１－４

上記の引用文献１に記載されるように、吸入用一酸化窒素は選択的肺血管拡張剤として周知のものである。

一方、引用文献２（Abstract等）には、左心室機能不全の患者に吸入用一酸化窒素を投与すると、左心充満圧の上昇に伴う肺血管抵抗の低下を引き起こし、有害事象が生ずる可能性があることが記載されている。

引用文献３（Abstract, "Treatment with iNO"等）には、新生児肺高血圧の治療に吸入用一酸化窒素が用いられるものの、左心室流路障害で特徴づけられる先天性心疾患や、重篤な左心室機能不全の患者に対しては、吸入用一酸化窒素による治療は禁忌であると記載されている。

引用文献４（p.71 左欄第13-15行）には、重篤な心疾患の患者に吸入用一酸化窒素を投与すると、肺水腫を引き起こす可能性があることが記載されている。

してみると、引用文献１－４の記載に基づき、有害事象の発生を避けるべく、選択的肺血管拡張剤の対象患者から、先在性左心室機能障害を有する患者を除外することは当業者が容易に想到し得たことである。

そして、請求項１－１４に係る発明が引用文献１－４の記載からみて格別顕著な効果を奏するとも認められない。

Ｂ.

・理由　３

（１）

・請求項　7

「ＰＡＰｍ」、「ＰＣＷＰ」、「ＰＶＲＩ」は略語であり、その意味が不明である。

（２）

・請求項　１０

「ほぼ」なる記載は発明の範囲を不明確とするものであって、特許請求の範囲の記載として適切でない。

引　用　文　献　等　一　覧

１．Inglessis, I. et al., Journal of the American College of Cardiology, ２００４年, Vol.44, No.4, p.793-798

２．Loh, E. et al., Circulation, １９９４年, 90, p.2780-2785

３．Steinhorn, R.H. et al., Pulmonary Hypertension, Persistent-Newborn, emedicine, Updated Apr 19, 2007 [http://emedicine.medscape.com/article/898437-overview]

４．BOCCHI, E.A. et al., The American Journal of Cardiology, １９９４年, Vol.74, p.70-72

（注）法律又は契約等の制限により、提示した非特許文献の一部又は全てが送付されない場合があります。

先行技術文献調査結果の記録

・調査した分野　　ＩＰＣ　Ａ６１Ｋ３３／００

この拒絶理由通知の内容に関するお問い合わせ、または面接のご希望がございましたら下記までご連絡下さい。

特許審査第三部医療　高橋樹理

ＴＥＬ．０３（３５８１）１１０１　内線３４５０

ＦＡＸ．０３（３５８１）１３４２

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE (USPTO)	
Application Serial Number	TBD
Confirmation Number	1376
Filing Date	Herein
Title of Application	Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension
First Named Inventor	James S. Baldassarre
Assignee	Ikaria, Inc.
Group Art Unit	1614
Examiner	TBD
Attorney Docket Number	I001-0002USC1

ACCELERATED EXAMINATION SUPPORT DOCUMENT

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

This Accelerated Examination Support Document (AESD) is submitted in support of the Petition for Accelerated Examination filed herewith.

Claims 1-19 are currently pending in the continuation application. A listing of the claims starts on page 2 herein.

The remaining sections of the AESD begin on page 6. Consideration and grant of the Petition to Accelerate Examination is respectfully requested.

CLAIMS

1. A method of reducing one or more adverse events or serious adverse events in an intended patient population comprising neonates or near-term neonates in need of being treated with inhalation of nitric oxide comprising excluding from such treatment anyone in the intended patient population having pre-existing left ventricular dysfunction.

2. The method of claim 1, wherein anyone in the intended patient population further has a pulmonary capillary wedge pressure greater than 20 mm Hg.

3. The method of claim 1, wherein the treatment further comprises inhalation of oxygen.

4. The method of claim 1, wherein the treatment is delivered using a ventilator.

5. The method of claim 1, wherein anyone in the intended patient population having pre-existing left ventricular dysfunction also having one or more conditions selected from diastolic dysfunction, hypertensive cardiomyopathy, systolic dysfunction, ischemic cardiomyopathy, viral cardiomyopathy, idiopathic cardiomyopathy, autoimmune disease related cardiomyopathy, drug-related cardiomyopathy, toxin-related cardiomyopathy, structural heart disease, valvular heart disease, congenital heart disease, idiopathic pulmonary arterial hypertension and pulmonary hypertension cardiomyopathy, and associations thereof.

6. The method of claim 1, wherein the intended patient population are at risk of one or more adverse events or serious adverse events selected from pulmonary edema, hypotension, cardiac arrest, electrocardiogram changes, hypoxemia, hypoxia and bradycardia, and, associations thereof.

7. The method of claim 1, further comprising reducing left ventricular afterload to minimize or reduce the risk of the occurrence of an adverse event or serious adverse event being pulmonary edema.

8. The method of claim 7, wherein the left ventricular afterload is minimized or reduced by administering a pharmaceutical dosage form comprising nitroglycerin or calcium channel blocker.

9. The method of claim 7, wherein the left ventricular afterload is minimized or reduced using an intra-aortic balloon pump.

10. The method of claim 1, wherein the intended patient population in need of being treated with the inhalation of nitric oxide has one or more of idiopathic pulmonary arterial hypertension characterized by PAPm > 25 mm Hg at rest, PCWP $\leq$ 15 mm Hg, and, a PVRI > 3 $u \cdot m^2$; congenital heart disease with pulmonary hypertension repaired and unrepaired characterized by PAPm > 25 mm Hg at rest and PVRI > 3 $u \cdot m^2$; cardiomyopathy characterized by PAPm > 25 mm Hg at rest and PVRI > 3 $u \cdot m^2$; or, the patient is scheduled to undergo right heart catheterization to assess pulmonary vasoreactivity by acute pulmonary vasodilation testing.

11. A method of reducing the risk or preventing the occurrence, in a patient being a neonate or near-term neonate of one or more adverse events or serious adverse events associated with a medical treatment comprising inhalation of nitric oxide, said method comprising:

a. identifying a patient eligible for inhalation of nitric oxide treatment;

b. determining if said patient has pre-existing left ventricular dysfunction; and,

c. administering said medical treatment if said patient does not have pre-existing left ventricular dysfunction;

thereby reducing the risk or preventing the occurrence of the adverse event or serious adverse event associated with said medical treatment.

12. The method of claim 11, wherein said patient further exhibits a pulmonary capillary wedge pressure greater than 20 mm Hg.

13. The method of claim 11, wherein the patient who has pre-existing left ventricular dysfunction has one or more conditions selected from diastolic dysfunction, hypertensive cardiomyopathy, systolic dysfunction, ischemic cardiomyopathy, viral cardiomyopathy, idiopathic cardiomyopathy, autoimmune disease related cardiomyopathy, side effects due to drug-related cardiomyopathy, side effects due to toxin-related cardiomyopathy, structural heart disease, valvular heart disease, congenital heart disease, idiopathic pulmonary arterial hypertension, pulmonary hypertension and cardiomyopathy, and associations thereof.

14. The method of claim 12, wherein the patient who has pre-existing left ventricular dysfunction has one or more conditions selected from diastolic dysfunction, hypertensive cardiomyopathy, systolic dysfunction, ischemic cardiomyopathy, viral cardiomyopathy, idiopathic cardiomyopathy, autoimmune disease related cardiomyopathy, side effects due to drug-related cardiomyopathy, side effects due to toxin-related cardiomyopathy, structural heart disease, valvular heart disease, congenital heart disease, idiopathic pulmonary arterial hypertension, pulmonary hypertension and cardiomyopathy, or associations thereof.

15. The method of claim 11, wherein the medical treatment further comprises inhalation of oxygen.

16. The method of claim 11, wherein the treatment is delivered using a ventilator.

17. The method of claim 11, further comprising reducing left ventricular afterload to minimize or reduce the risk of the occurrence of an adverse event or serious adverse event being pulmonary edema.

18. The method of claim 17, wherein the left ventricular afterload is minimized or reduced by administering a pharmaceutical dosage form comprising nitroglycerin or calcium channel blocker.

19. The method of claim 17, wherein the left ventricular afterload is minimized or reduced using an intra-aortic balloon pump.

9(A) References Deemed Most Closely Related

An Information Disclosure Statement in compliance with 37 CFR 1.98 has been filed herewith citing each of the following references deemed most closely related to the subject matter of the claims. The references listed in the IDS submitted herewith but not listed in this Petition are not closely related to the claimed invention particularly as compared to the references listed and discussed herein.

List of Most Closely Related References

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Adatia I et al., Inhaled Nitric Oxide and Hemodynamic Evaluation of Patients With Pulmonary Hypertension Before Transplantation, JADD, Vol. 25, No. 7, June 1995, pp. 1656-64. ("Adatia").

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9(B) Identification of Limitations Disclosed by References

AAP:

In August 2000, the Committee on Fetus and Newborn of the American Academy of Pediatrics issued a report on the use of iNO in infants. A relevant portion states:

> iNO should be administered using FDA-approved devices that are capable of administering iNO in constant concentration ranges in parts per million or less throughout the respiratory cycle. Infants who receive iNO therapy should be monitored according to institutionally derived protocols designed to avoid the potential toxic effects associated with iNO administration. These effects include methemoglobinemia (secondary to excess nitric oxide concentrations), direct pulmonary injury (attributable to excess levels of nitrogen dioxide), and ambient air contamination.

(P. 344, 2nd col.). AAP also lists seven RECOMMENDATIONS. (Pp. 344-345). However, AAP is completely silent respecting excluding from iNO treatment any neonate or near-term neonate patient diagnosed with pre-existing left ventricular dysfunction.

Lipshultz:

Lipshultz teaches that data or information gleaned from iNO studies in adults does not correlate or is otherwise probative of iNO studies in neonates or near-term neonates. In other words, near-term neonates with ventricular dysfunction must be diagnosed, understood, and treated differently than adult patients diagnosed with ventricular dysfunction. Relevant statements are found in the abstract:

> Many changing developmental properties of the pediatric myocardium and differences in the etiologies of ventricular dysfunction in children compared with adults [exist] ... invalidating the concept that children can safely be considered small adults for the purpose of understanding heart failure pathophysiology and treatment.

At page 2, the author states:

> The disease processes resulting in ventricular dysfunction are often different in children than adults. Many pediatric conditions have no close analogies in the adult ... [hence] the effects of intervention may be unlike those seen in adults.

And, at page 5, the author states:

> when trying to understand the proper therapy for children with ventricular dysfunction it is usually important not to view the child as a small adult and extrapolate the effects of ventricular dysfunction therapy for adult ischemia or post-infarction patients to the child where a multitude of non-ischemic, non post-infarction etiologies exist.

NINOS:

> At page 597 under "Conclusions" it states:
>
> Nitric oxide therapy reduced the use of extracorporeal membrane oxygenation, but had no apparent effect of mortality, in critically ill infants with hypoxic respiratory failure.

As set forth in the "Results" section on page 597, the study included 121 infants in the control group and 114 infants in the nitric oxide group. Left ventricular dysfunction was not mentioned.

As to patient eligibility, NINOS states:

> Infants born at 34 or more weeks of gestation who required assisted ventilation for hypoxic respiratory failure and had an oxygenation index of at least 25 on two measurements made at least 15 minutes apart were eligible for the trial.
> Infants were considered ineligible for the study if they were more than 14 days old, had a congenital heart disease, or if it had been decided not to provide full treatment.

(P. 598 under "Study Patients").

Hayward 1996:

The ten patients (19 to 59 years old) in this study had severe LV dysfunction and secondary pulmonary hypertension. (See p. 81 under "Methods" and Results" headings). iNO was administered in 10, 20 and 40 ppm doses. (Id. at 2nd col.). The study concludes stating:

> Our results confirm the safety and utility of INO in short-term assessment of pulmonary hypertension in patients with severe cardiac impairment. The possibility of worsening cardiac function in some patients is worrisome, however, and suggests that INO should be used cautiously in such patients and only in combination with other treatments that have been shown to improve LV function. Safety guidelines for the use of INO were recently formulated. We recommend that these guidelines be expanded to include caution regarding the use of INO in patients with severe LV dysfunction. Further study of the haemodynamic effects of INO on the left ventricle is needed.

(P. 84).

Hayward 1997:

This study was conducted in eleven adults being 51-69 years old with normal LV function. (P. 49, under "Methods" heading). The objective of the study was to determine the effects of iNO on load-independent indexes of normal human LV function. (Id. under "Objectives" heading). The results were that iNO had no effect on steady state LV pressure, volume, contractility duration, active relaxation, diastolic compliance or PVR. (Id. under "Results" heading). Thus, it was concluded that 20 ppm of iNO does not significantly affect normal LV function. (Id. under "Conclusions" heading).

Roberts:

The study included 30 newborn infants having "severe hypoxemia even though they were receiving mechanical ventilation at an FiO_2 of 1.0" (p. 606 under "Criteria for Eligibility") to determine whether iNO decreases severe hypoxemia in infants with persistent pulmonary hypertension. (See Abstract and Results, p. 605). The study concluded that "[i]nhaled nitric oxide improves systemic oxygenation in infants with persistent pulmonary hypertension and may reduce the need for more invasive treatments." (See Conclusions, p. 605).

Roberts further states under the "Criteria for Eligibility" heading:

> Infants were excluded from the study if they had any of the following: previous treatment with extracorporeal membrane oxygenation or high-frequency oscillatory or jet ventilation, a congenital diaphragmatic hernia or suspected lung hypolasia, structural cardiac lesions (other than a patent ductus arteriosus), uncorrected hypotension (a mean aortic pressure below 40 mm Hg) or polycythemia (an arterial hematocrit of at least 70 percent), an unevacuated pneumothorax, or a phenotype consistent with a lethal chromosomal abnormality. Since infants who have received exogenous surfactant without sustain increases in systemic oxygenation have responses to inhaled nitric oxide similar to those of infants not previously treated with surfactant, they were not excluded from the study.

Loh:

This is a study of 19 patients with an average age of 52 +/- 3 years. (See p. 2780 under "Study Population" heading). These adult patients suffered from ischemic cardiomyapathy (heart failure due to coronary artery disease and resultant partial cardiac muscle death) and idiopathic dilated cardiomyopathy. (Id.). Fourteen of the patients were diagnosed with left ventricular dysfunction. (See p. 2780 under "Methods and Results" heading).

Loh discloses:

> The most prominent hemodynamic effect of NO inhalation was the increase in pulmonary artery wedge pressure (median increase 26%).
> Thus, more severe LV dysfunction (as evidenced by higher left heart filling pressures, lower stroke volume, and larger LV cavity size) was present in the patients who had the largest increases in pulmonary artery wedge pressure with inhaled NO.

(P. 2782 under "Hemodynamic Determinants of an Increase in Pulmonary Artery Wedge Pressure With Inhaled NO" heading).

Loh further discloses:

> The major finding of this study is that in patients with reactive pulmonary arterial hypertension secondary to LV failure, inhalation of NO causes reciprocal changes in the PVR (decrease) and LV filling pressure (increase). In contrast, in patients with LV failure, we found that inhalation of NO is associated not with a decrease in pulmonary artery pressure, but rather, with an increase in LV filling pressure that accounts for the decrease in PVR.

(P. 2783 under "Discussion" heading).

Inglessis 2004:

This is a study of 13 patients with an average age of 65 +/- 3 years. (See p. 793 under "Methods" heading). The objective of the study was to see if iNO improved "cardiac performance in patients with RVMI and CS." (See p. 794).

Under the "Methods" heading at page 794, the reference discloses:

> Patients were then included for further study if their right atrial (RA) pressure was >10 mm Hg, their PCWP was no >5 mm Hg higher than the RA pressure, and their CI was <2.5 l/min/m^2. Patients were excluded from the study if they had severe pulmonary edema (PCWP >25 mm Hg; n=4), mechanical complications of MI requiring urgent surgical correction (N=0), severe mitral or aortic valvular disease (n=1), persistent hemodynamically significant tachyarrhythmias (n=1), or a history of clinically significant pulmonary disease (n=0).

The reference further discloses:

> In this study, PCWP did not change during NO inhalation by RVMI patients, as has been previously observed during administration to patients with severe LV systolic dysfunction. In patients with severe LV systolic dysfunction, which is usually accompanied by poor diastolic ventricular compliance, breathing NO is thought to increase pulmonary venous return, resulting in an increase in LV filling pressure. The RVMI patients in this study had primarily RV systolic and diastolic function, and the degree of LV dysfunction was not as severe as in those patients in whom the PCWP has been reported to increase during NO inhalation.

(P. 797, 2nd col.).

Inglessis 2005:

In a reply, the author states "[p]atients with severe LV systolic function should be monitored carefully during chronic NO inhalation because of the possibility of their developing pulmonary venous hypertension." (P. 965, 2nd col.).

Bocchi:

This study included 3 patients ages 40, 41, and 52 years old suffering from either ischemic or idiopathic cardiomyapathy. (P. 70, 1st col.). All three adults had severe pulmonary HTN and refractory heart failure and were candidates for cardiac transplantation. (Id.) All three patients were treated with iNO.

The reference discloses:

> Results of this investigation demonstrate that acute inhaled nitric oxide produces rapid pulmonary vasodilation in the absence of hypoxia in patients with severe heart failure. However, nitric oxide inhalation was associated with an increment in pulmonary pressure, mainly pulmonary wedge pressure, and an improvement in cardiac output. In addition, inhaled nitric oxide may lead to pulmonary edema in patients with severe heart failure.

(P. 71, 1st col.).

Cujec:

This is a case study involving 33 adults with a mean age of 69 +/- 11 years, most of whom had significant valvular disease and dysfunctional LV characterized by a reduced ejection fraction. (P. 816 under "Patients" heading, and p. 819 under "Results" heading).

Cujec concludes at page 823 stating:

> We found in a randomized and blinded trial that the reduction in pulmonary artery systolic pressure following nitric oxide inhalation depends on the pre-existing LVEF. Our results in patients with a broader mix of cardiac pathology confirm previous case series. These observations suggest further limitations for the clinical role of inhaled nitric oxide. We postulate that in patients with the least cardiac reserve, decreasing venous but not arterial pulmonary vascular resistance may cause an increase in regional pulmonary edema. Through reflex mechanisms, this could further impair cardiopulmonary function resulting in cardiac decompensation, worsening pulmonary hypertension and generalized pulmonary edema. This study cautions against the ubiquitous use of inhaled nitric oxide in the treatment of all critically ill patients. Nitric oxide is not just a pulmonary vasodilator but has profound effects on many other systems. The adverse effects of nitric oxide may become most evident in patients with the least cardiac reserve.

Rosales:

This is a case report of a one-month old neonate that developed rebound pulmonary hypertension after receiving iNO. (See Abstract at p. 224). The infant patient was diagnosed with total anomalous pulmonary venous return (three pulmonary veins draining into the portal system below the diaphragm and the remaining upper left pulmonary vein draining into the innominate vein). (Id.).

This infant underwent surgical correction and in the post operative period received iNO. (See p. 225, 1st col.). iNO was discontinued based on the rationale that the episode of pulmonary HTN may have been caused by left atrial hypertension secondary to a sudden increase in pulmonary blood flow into a non-compliant left atrium and ventricle due in part to the redirection of blood flow from the surgical correction. (See p. 225, 2nd col.).

Argenziano:

This study in pigs resulted in the following conclusion:

> In conclusion, we have reproduced, in a porcine model of heart failure and pulmonary hypertension, the constellation of clinically observed hemodynamic responses to inhaled NO therapy, including dose-dependent decreases in pulmonary arterial pressure and PVR and increases in LVEDP. Furthermore, determination of the ESPVR, PRSW, EDPVR, and T in these animals has demonstrated no effect of inhaled NO on myocardial contractility or relaxation. An alternative explanation that has been proposed on theoretical grounds is that volume shifts caused by pulmonary vasodilation are responsible for clinically observed elevations in left atrial pressure and may also explain why patients with preexisting ventricular dysfunction are at greatest risk for these pressure elevations. Although clinical validation of our findings in humans is necessary and is the subject of current investigations, an understanding of this mechanism may lead to strategies allowing the safe use of inhaled NO in heart failure, perhaps by adjunctive vasodilator therapy.

(P. 707).

Steinhorn 2007:

This is a review article of persistent pulmonary HTN. It is a general discussion and review, not a clinical study. No data is provided. It points out that iNO is contraindicated in congenital heart disease (e.g., interrupted AO arch, critical AO stenosis, and hypolplastic LV) and severe LV dysfunction.

Under the heading "Treatment with iNO," it states:

> Treatment with iNO for newborns with an OI>25. Nitric oxide (NO) is an endothelial-derived gas signaling molecule that relaxes vascular smooth muscle and that can be delivered to the lung by means of an inhalation device (INOVent; Datex-Ohmeda Ind, Madison, WI).
> In 2 large randomized trials, NO reduced the need for ECMO support by approximately 40%.
> Contraindications to iNO include congenital heart disease characterized by left ventricular outflow tract obstruction (eg, interrupted aortic arch, critical aortic stenosis, hypoplastic left heart syndrome) and severe left ventricular dysfunction.

Krasuski:

This reference reports the results of a clinical study in forty-two adult patients (26 to 77 years old) having pulmonary hypertension during cardiac catheterization and receiving iNO. (See Abstract, p. 2204). The reference concludes that

> Nitric oxide is a safe and effective screening agent for pulmonary vasoreactivity. Regardless of etiology of pulmonary hypertension, pulmonary vasoreactivity is frequently demonstrated with the use of NO. Right ventricular diastolic dysfunction may predict a poor vasodilator response.

(Id. under "Conclusions" heading).

Semigran:

This study included 16 adults (13 men and 3 women) having a mean age of 51 ± 2 years each having class III or IV heart failure and being considered for heart transplantation. (See p. 983, 1st col.). No patient had a history of primary pulmonary disease, and pulmonary function testing was consistent with chronic left heart failure. (Id.). The patients were treated with digoxin, diuretic drugs, vasodilators and amiodarone. (Id.) iNO was administered at 20, 40 and 80 ppm. (Id. at 2nd col.).

The reference concludes stating:

> Inhaled nitric oxide is a selective pulmonary vasodilator in patients with severe chronic heart failure. The selectivity of inhaled nitric oxide for the pulmonary circulation offers a potential advantage over nonselective vasodilators such as nitroprusside in the identification of reversible pulmonary vasoconstriction in potential heart transplant recipients. Nitric oxide increases left ventricular filling pressure in patients with severe heart failure by an unknown mechanism.

(P. 982 under "Conclusions" heading).

Dickstein:

The reference teaches mathematical (see Appendix at p. 720) and electric circuit (see Figure 1 at p. 717) models of a cardiovascular system as "time varying elastances: the pulmonary and systemic vascular systems were each modeled as a series of resistive and compliance elements." (P. 715 under "Methods" heading).

The reference concludes stating:

> Pulmonary vasodilation by itself can lead to an increase in pulmonary venous pressure that is mediated by shifts of blood between arterial and venous compartments of the pulmonary bed. Furthermore, impairment in ventricular contractile state by itself has relatively little effect on pulmonary venous pressure. The magnitude of the increase in pulmonary venous pressure is largely determined by the volume status and the initial value of pulmonary vascular resistance.

(P. 715 under "Conclusions" heading).

Dickstein further discloses:

> The present analysis suggests that it is not necessary for this agent [i.e., nitric oxide] to work as a negative inotrope to cause pulmonary venous pressure to rise: its pulmonary vasodilating actions alone are sufficient to explain why patients with preexisting heart failure are at greatest risk for pulmonary edema.

(P. 719, 2nd col.).

Henrichsen:

This reference is a letter to the editor of journal reporting iNO treatment of a baby born at 38 weeks of gestation diagnosed with persistent pulmonary hypertension of the newborn (PPHN) and severe left ventricular dysfunction. The baby was treated with 20 ppm iNO which "resulted in an immediate fall in the mean systemic arterial blood pressure from 48 to 35 mm Hg, which reversed when the NO therapy was discontinued." In other words, the iNO caused systemic hypotension.

As second iNO treatment thirty hours later "resulted in a marked improvement in oxygenation, from an arterial oxygen tension to 16 to 420 mm Hg without a change in the systemic arterial blood pressure."

Ovodov:

The review article discusses various clinical studies of PPHN using iNO. (P. 95, 2nd col.). In particular, the reference cites the NINOS trial. (Id.) It concludes that "[s]afety of low-dose inhaled nitric oxide in newborns has been suggested by several studies" and that "there are no reports of any related adverse clinical manifestations." (P. 96, 1st col.).

Adatia:

This reference reports the results of a study involving 11 patients ranging in age from 0.7 to 27 years with a median of 13 years diagnosed with pulmonary hypertension. (P. 1656, 2nd col.). Some of the patients were diagnosed with "severe left ventricular failure despite optimal medical management with digoxin, diuretic drugs and, when appropriate, maximal afterload reduction therapy." (P. 1657, 1st col.).

The reference concludes stating:

> These preliminary observations suggest that nitric oxide is a potent pulmonary vasodilator with minimal systemic effects. It may be useful in discriminating patients needing combined heart and lung transplantation from those requiring exchange of the heart alone.

(P. 1656 under Conclusions heading).

Findlay:

This reference is a case report concerning a 22-year old man treated with iNO where the patient had a "paradoxical response to inhaled nitric oxide, where a rise in mean pulmonary artery and pulmonary artery occlusion pressure and a fall in cardiac output and stroke volume occurred, in a young man with meningococcaemia." (P. 134, 1st col.).

9(C) Detailed Explanation of Patentability

None of the references disclose excluding from iNO treatment any patient in the patient population (comprising neonates or near-term neonates) that have been diagnosed as having pre-existing left ventricular dysfunction (LVD) in order to avoid adverse events or serious adverse events. (See independent claims 1 and 11). Thus, independent claims 1 and 11 are patentably novel and nonobvious over the listed most relevant references as well as the other references of record. Moreover, dependent claims 2-10 and 12-19 are patentably novel and nonobvious for at least the same reasons set forth herein respecting independent claims 1 and 11.

The AAP reference is highly relevant due to the prominence of the Pediatric Committee. The fact that it is silent respecting excluding from iNO treatment any infant patient diagnosed with pre-existing left ventricular function speaks louder than words.

Lipshultz teaches that data and information gleaned from iNO studies in adults do not correlate or are otherwise probative of iNO studies in children. Thus, the Hayward 1996 & 1997, Loh, Inglessis 2004 & 2005, Bocchi, Cujec, Krasuski, Findlay and Semigran references are not probative of the instantly claimed invention.

Pre-existing LVD is not mentioned in the NINOS reference involving infants. While the Roberts involves neonate patients, it fails to teach excluding such patients if they have been diagnosed with pre-existing LVD.

Rosales involves a one-month old neonate patient undergoing surgical correction and post operative iNO treatment. Rosales also fails to teach or suggest pre-existing LVD as exclusionary criteria for iNO treatment.

Argenziano is a pig study that also fails to teach or suggest pre-existing LVD as exclusionary criteria for iNO treatment.

Steinhorn 2007 is a general discussion and review. No data is provided. Therefore, Steinhorn 2007 is a non-enabling reference.

Dickstein is a "purely theoretic analysis of the impact of NO therapy on pulmonary venous pressure." (P. 719, 2nd col.). The reference fails to disclose any data to support this unpredictable science which is also not well understood, therefore, Dickstein is non-enabling prior art. The reference also teaches away from excluding a patient from being treated with iNO where the patient has been diagnosed with pre-

existing LVD. For example, the reference theorizes that increased volume causes the risk of adverse events stating:

> results of the present analysis would suggest that patients with heart failure are at increased risk for development of pulmonary edema during NO therapy because of the high effective volume status.

(P. 719, 2nd col.).

Henrichsen is a report of a single near-term neonate having PPHN and LVD that experienced systemic hypotension when treated with iNO, which is contrary to the accepted understanding that nitric oxide is a selective vasodilator, i.e., non-systemic. Moreover, the subsequent iNO treatment had a positive therapeutic outcome. Henrichsen fails to teach LVD as exclusionary criteria in the claimed patient population, and it teaches away from the invention by merely cautioning iNO treatment.

The instant claims are patentable over Ovodov, Adatia and Findlay at least because each reference fails to teach or suggest excluding the claimed patient population having LVD from being treated with iNO.

9(D) Concise Statement of Utility

The instantly claimed invention is eligible subject matter under 35 USC 101 for patentable utility in that the claims are generally directed to a method of excluding patients in need of being treated with inhaled nitric oxide. The purpose of such mandatory exclusion is to reduce the incidence of adverse events or serious adverse events. Patients in an intended patient population are excluded from such treatment (even though the inhaled nitric oxide treatment would be potentially beneficial to the patient) if the patient has pre-existing left ventricular dysfunction.

9(E) Showing of Support under 35 USC 112, First Paragraph

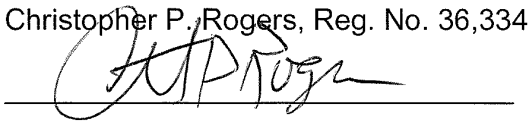
Support and antecedent basis for the claimed invention is found at least in the SUMMARY OF THE INVENTION as originally filed at pages 2-4 and ¶¶[0005]-[0020]. Enablement of the claimed invention is found at least in the DETAILED DESCRIPTION OF THE EXEMPLARY EMBODIMENTS at pages 4-13 and ¶¶[0021]-[0050] as well as in EXAMPLE1: INOT22 STUDY at pages 13-22 and ¶¶[0051]-[0069].

9(F) Identification of References Disqualified as Prior Art under 35 USC 103(c)

None of the cited references are disqualified as prior art under 35 USC 103(c).

Respectfully Submitted,

Christopher P. Rogers, Reg. No. 36,334



Date: 2010/06/21

Lee & Hayes, PLLC
601 W. Riverside Avenue, Suite 1400
Spokane, WA 99201

PTO/SB/01 (07-07)
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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☐ Declaration Submitted With Initial Filing **OR** ☒ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	135197.00084
First Named Inventor	James S. Baldassarre
COMPLETE IF KNOWN	
Application Number	12/494,598
Filing Date	Herewith
Art Unit	1614
Examiner Name	

I hereby declare that:

Each inventor's residence, mailing address, and citizenship are as stated below next to their name.

I believe the inventor(s) named below to be the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☒ was filed on (MM/DD/YYYY) 06/30/2009 as United States Application Number or PCT International Application Number 12/494,598 and was amended on (MM/DD/YYYY) _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

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DECLARATION — Utility or Design Patent Application

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF SOLE OR FIRST INVENTOR:	☐ A petition has been filed for this unsigned inventor
Given Name (first and middle [if any])	Family Name or Surname
James S.	Baldassarre
Inventor's Signature	Date
	7/28/09

Residence: City	State	Country	Citizenship
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145 Pebble Woods Dr			
City	State	Zip	Country
Doylestown	PA	18901	US

☑ Additional inventors or a legal representative are being named on the 1 supplemental sheet(s) PTO/SB/02A or 02LR attached hereto.

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DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet
Page ___ of ___

Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any])		Family Name or Surname	
Ralf		Rosskamp	
Inventor's Signature R. Ross Mroz			**Date** 7/28/09
Residence: City Chester	**State** NJ	**Country** US	**Citizenship** DE
Mailing Address 1 Byron Court			
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City Chester	**State** NJ	**ZIP** 07930	**Country** US

Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any])		Family Name or Surname	
Inventor's Signature			**Date**
Residence: City	**State**	**Country**	**Citizenship**
Mailing Address			
Mailing Address			
City	**State**	**ZIP**	**Country**

Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any])		Family Name or Surname	
Inventor's Signature			**Date**
Residence: City	**State**	**Country**	**Citizenship**
Mailing Address			
Mailing Address			
City	**State**	**ZIP**	**Country**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE (USPTO)	
Application Serial Number	TBD
Confirmation Number	TBD
Filing Date	Herein
Title of Application	Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension
First Named Inventor	James S. Baldassarre
Assignee	Ikaria, Inc.
Group Art Unit	TBD
Examiner	TBD
Attorney Docket Number	I001-0002USC1

To: Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

From: Christopher P. Rogers (Tel.; Fax 509-323-8979)
Customer Number: 49584
Lee & Hayes, PLLC
601 W. Riverside Avenue, Suite 1400
Spokane, WA 99201

Fees will be paid by credit card through the EFS Web; however the Commissioner is hereby authorized to charge any deficiency of fees and credit any overpayments to Deposit Account Number 12-0769.

Respectfully Submitted,

Dated: 2010/06/21

By: _____
Christopher P. Rogers
Reg. No. 36,334

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE (USPTO)	
Priority Application Serial No	12/494,598
Priority Filing Date	06/30/2006
Title of Application	Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension
First Named Inventor	James S. Baldassarre
Priority Group Art Unit	1614
Priority Examiner	TBD
Attorney Docket Number	I001-0002USC1

INFORMATION DISCLOSURE STATEMENT

The citations listed are submitted in compliance with the duty of disclosure defined in 37 CFR §1.56. Copies of the cited references were cited or submitted with the priority application and are therefore not submitted herewith.

The Examiner is requested to make these citations of official record in this application.

Respectfully Submitted,

Date: 2010/06/21

By: _____
Christopher P. Rogers
Reg. No. 36,334

PTO/SB/08a (08-03)
Approved for use through 07/31/2006. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99**)		
	Application Number	
	Filing Date	
	First Named Inventor	James S. Baldassarre
	Art Unit	
	Examiner Name	
	Attorney Docket Number	I001-0002USC1

U.S.PATENTS						
Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5873359		1999-02-23	Zapol, ; et al.	
	2	6063407		2000-05-16	Zapol, ; et al.	
	3	6601580		2003-08-05	Bloch, ; et al.	
	4	7557087		2009-07-07	Rothbard, ; et al.	

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	1	20040106954	A1	2004-06-03	Whitehurst, Todd K.; et al.	
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	3	20090029371	A1	2009-01-29	Elliott; C. Gregory	

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	Attorney Docket Number	I001-0002USC1

	4	20090149541	A1	2009-06-11	Stark et al.	
	5	20090176772	A1	2009-07-09	Blackburn et al.	

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FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	EP1682672;(A1)			2006-07-26	COUNCIL SCIENT IND RES [IN]+ (COUNCIL O		☐
	2	WO2005004884;(A2)			2005-01-20	US GOVERNMENT [US]; UN		☐
	3	WO2006127907;(A2)			2006-11-30	MASSACHUSETTS INST TECHNOLOGY [US];		☐
	4	WO2010019540;(A1)			2010-02-18	NOVARTIS AG [CH]; PASC		☐

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NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
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	Examiner Name	
	Attorney Docket Number	I001-0002USC1

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	Filing Date	
	First Named Inventor	James S. Baldassarre
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	Examiner Name	
	Attorney Docket Number	I001-0002USC1

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	Examiner Name	
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	58	Weinberger B et al., "The Toxicology of Inhaled Nitric Oxide", Toxicological Sciences, 59, pp. 5-16 (2001).	☐
	59	Weinberger, et al., "Nitric Oxide in the lung: therapeutic and cellular mechanisms of action", Pharmacology & Therapeutics 84 (1999) 401-411	☐
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CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement.

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SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

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TREATMENT OF SPECIFIC CARDIOVASCULAR CONDITIONS WITH NITRITE

Abstract:

Abstract of WO2005004884

It has been surprisingly discovered that administration of nitrite to subjects causes a reduction in blood pressure and an increase in blood flow to tissues. The effect is particularly beneficial, for example, to tissues in regions of low oxygen tension. This discovery provides useful treatments to regulate a subject's blood pressure and blood flow, for example, by the administration of nitrite salts. Provided herein are methods of administering a pharmaceutically-acceptable nitrite salt to a subject, for treating, preventing or ameliorating a condition selected from : (a) ischemia-reperfusion injury (e.g., hepatic or cardiac or brain ischemia-reperfusion injury); (b) pulmonary hypertension (e.g., neonatal pulmonary hypertension); or (c) cerebral artery vasospasm. Data supplied from the esp@cenet database - Worldwide c70

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(54) Title: TREATMENT OF SPECIFIC CARDIOVASCULAR CONDITIONS WITH NITRITE

(57) Abstract: It has been surprisingly discovered that administration of nitrite to subjects causes a reduction in blood pressure and an increase in blood flow to tissues. The effect is particularly beneficial, for example, to tissues in regions of low oxygen tension. This discovery provides useful treatments to regulate a subject's blood pressure and blood flow, for example, by the administration of nitrite salts. Provided herein are methods of administering a pharmaceutically-acceptable nitrite salt to a subject, for treating, preventing or ameliorating a condition selected from : (a) ischemia-reperfusion injury (e.g., hepatic or cardiac or brain ischemia-reperfusion injury); (b) pulmonary hypertension (e.g., neonatal pulmonary hypertension); or (c) cerebral artery vasospasm.

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TREATMENT OF SPECIFIC CARDIOVASCULAR CONDITIONS WITH NITRITE

Cross Reference to Related Applications

This application claims the benefit of U.S. Provisional Application No. 60/485,959, filed July 9, 2003, and No. 60/511,244, filed October 14, 2003, both of which are incorporated herein by reference in their entirety.

Government Interest Statement

Aspects of this invention were developed with government support under Grant Nos. HL58091 (D.B.K.-S.), and HL70146 (R.P.P.), both awarded by the National Institutes of Health. The government has certain rights in aspects of the invention. The government also may have certain rights in the invention due to at least one inventor's employment by the National Institutes of Health.

Background of the Disclosure

The last decade has seen an increase in the understanding of the critical role nitric oxide as a blood vessel dilator contributing to the regulation of blood flow and cardiovascular homeostasis. Nitric oxide may be oxidized in blood to nitrite (NO_2-), an anion considered to be an inert metabolic end product of such nitric oxide oxidation. *In vivo* plasma levels of nitrite have been reported to range from 150 to 1000 nM, and the nitrite concentration in aortic ring tissue has been reported to be in excess of 10,000 nM (Rodriguez *et al.*, *Proc Natl Acad Sci U S A, 100*, 336-41, 2003; Gladwin *et al.*, *Proc Natl Acad Sci U S A, 97*, 9943-8, 2000; and Rassaf *et al.*, *Nat Med, 9*, 481-3, 2003). This potential storage pool for NO is in excess of plasma S-nitrosothiols, which have been reported to be less than 10 nM in human plasma (Rassaf *et al.*, *Nat Med, 9*, 481-3, 2003; Rassaf *et al.*, *Free Radic Biol Med, 33*, 1590-6, 2002; Rassaf *et al.*, *J Clin Invest, 109*, 1241-8, 2002; and Schechter *et al.*, *J Clin Invest, 109*, 1149-51, 2002). Mechanisms have been proposed for the *in vivo* conversion of nitrite to NO, for example, by enzymatic reduction by xanthine oxidoreductase or by non-enzymatic disproportionation/acidic reduction (Millar *et al.*, *Biochem Soc Trans, 25*, 528S, 1997; Millar *et al.*, *FEBS Lett, 427*, 225-8, 1998; Godber *et al.*, *J Biol Chem, 275*, 7757-63, 2000; Zhang *et al.*, *Biochem Biophys Res Commun, 249*, 767-72, 1998 [published erratum appears in *Biochem Biophys Res Commun 251*, 667, 1998]; Li *et al.*, *J Biol Chem, 276*, 24482-9, 2001; Li *et al.*, *Biochemistry, 42*, 1150-9, 2003; Zweier *et al.*, *Nat Med, 1*, 804-9, 1995; Zweier *et al.*, *Biochim Biophys Acta, 1411*, 250-62, 1999; and Samouilov *et al.*, *Arch Biochem Biophys, 357*:1-7, 1998).

Arterial-to-venous gradients of nitrite across the human forearm at rest and during regional NO synthase inhibition have been observed, with increased consumption of nitrite occurring with exercise (Gladwin *et al.*, *Proc Natl Acad Sci U S A, 97*, 9943-8, 2000; Gladwin *et al.*, *Proc Natl Acad Sci USA, 97*, 11482-11487, 2000; and Cicinelli *et al.*, *Clin Physiol, 19*:440-2, 1999). Kelm and colleagues have reported that large artery-to-vein gradients of nitrite form across the human forearm during NO synthase inhibition (Lauer *et al.*, *Proc Natl Acad Sci USA, 98*, 12814-9, 2001). Unlike the more simple case of oxygen extraction across a vascular bed, nitrite may be both consumed, as

evidenced by artery-to-vein gradients during NO synthase inhibition and exercise, and produced in the vascular bed by endothelial nitric oxide synthase-derived NO reactions with oxygen.

At high concentrations, nitrite has been reported to be a vasodilator *in vitro* (Ignarro *et al.*, *Biochim Biophys Acta, 631*, 221-31, 1980; Ignarro *et al.*, *J Pharmacol Exp Ther, 218*, 739-49, 1981; Moulds *et al.*, *Br J Clin Pharmacol, 11*, 57-61, 1981; Gruetter *et al.*, *J Pharmacol Exp Ther, 219*, 181-6, 1981; Matsunaga *et al.*, *J Pharmacol Exp Ther, 248*, 687-95, 1989; and Laustiola *et al.*, *Pharmacol Toxicol, 68*, 60-3, 1991). The levels of nitrite shown to vasodilate *in vitro* have always been in excess of 100,000 nM (100 μM) and usually at millimolar concentrations.

Consistent with the high concentrations of nitrite required to vasodilate *in vitro*, when Lauer and colleagues infused nitrite into the forearm circulation of human subjects, they reported no vasodilatory effects, even with concentrations of 200 μM in the forearm (Lauer *et al.*, *Proc Natl Acad Sci USA, 98*, 12814-9, 2001). Lauer *et al.* reported that a "complete lack of vasodilator activity of intraartierial infusions of nitrite clearly rules out any role for this metabolite in NO delivery" and concluded that "physiological levels of nitrite are vasodilator-inactive." Furthermore, Rassaf and colleagues also failed to find a vasodilatory effect in humans following infusion of nitrite (Rassaf *et al.*, *J Clin Invest, 109*, 1241-8, 2002). Thus, *in vivo* studies have concluded that physiological levels of nitrites do not serve as a source for NO, and that physiological levels of nitrites do not have a role in regulating blood pressure.

Historically, nitrite has been used as a treatment for cyanide poisoning. High concentrations are infused into a subject suffering cyanide poisoning in order to oxidize hemoglobin to methemoglobin, which will bind cyanide. These high concentrations of nitrite produce clinically significant methemoglobinemia, potentially decreasing oxygen delivery. While these high concentrations of nitrite have been shown to decrease blood pressure in humans, the amount of methemoglobin formed precluded a use for nitrite in the treatment of other medical conditions.

Therefore, the state of the art was that nitrite was not a significant vasodilator at concentrations below 100 μM *in vitro*, and even when infused into humans at concentrations of 200 μM in the forearm. It was also the state of the art that nitrite was not converted to nitric oxide in the human blood stream.

Summary of the Disclosure

It has been surprisingly discovered that administration of pharmaceutically-acceptable salts of nitrite is useful in the regulation of the cardiovascular system. It has also been surprisingly discovered that nitrite is reduced to nitric oxide *in vivo*, and that the nitric oxide produced thereby is an effective vasodilator. These effects surprisingly occur at doses that do not produce clinically significant methemoglobinemia. These discoveries now enable methods to prevent and treat conditions associated with the cardiovascular system, for example, high blood pressure, pulmonary hypertension, cerebral vasospasm and tissue ischemia-reperfusion injury. These discoveries also provide methods to increase blood flow to tissues, for example, to tissues in regions of low oxygen tension. It is particularly surprising that the nitrite does not need to be applied in an acidified

condition in order for it to be effective in regulating the cardiovascular system, and more particularly to act as a vasodilator *in vivo*.

It has now been surprisingly discovered by the inventors that nitrite can serve as a vasodilator in humans at much lower concentrations (as low as 0.9 μM) than have been used in the past for cyanide poisoning. The mechanism is believed to involve a reaction of nitrite with deoxygenated hemoglobin and red blood cells, to produce the vasodilating gas nitric oxide. This potent biological effect is observed at doses of nitrite that do not produce clinically significant methemoglobininemia (for instance, less than 20%, more preferably less than 5% methemoglobin in the subject).

It has been discovered that nitrite is converted to nitric oxide *in vivo*, and that the nitric oxide produced thereby is an effective vasodilator. Further, it has been surprisingly discovered that administration of nitrite, for instance a pharmaceutically-acceptable salt of nitrite, to a subject causes a reduction in blood pressure and an increase in blood flow to tissues, for example, to tissues in regions of low oxygen tension. These discoveries now enable useful methods to regulate the cardiovascular system, for instance to prevent and treat malconditions associated with the cardiovascular system, for example, high blood pressure, or organs, tissues, or systems suffering a lack of or inadequate blood flow. Non-limiting examples of contemplated malconditions include stroke, heart disease, kidney disease and failure, eye damage including hypertensive retinopathy, diabetes, and migraines.

In one example embodiment, the present disclosure provides a method for decreasing a subject's blood pressure or increasing blood flow, including in a particular embodiment administering to the subject sodium nitrite at about 36 μmoles per minute into the forearm brachial artery.

The present disclosure additionally provides a method for increasing blood flow to a tissue of a subject, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite, such as a salt thereof, so as to increase blood flow to a tissue of the subject. The blood flow may be specifically increased in tissues in regions of low oxygen tension. The present disclosure also provides a method for decreasing a subject's blood pressure, comprising administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to decrease the subject's blood pressure.

The present disclosure further provides a method for treating a subject having a condition associated with elevated blood pressure, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the elevated blood pressure.

Also provided is a method for treating a subject having a hemolytic condition, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the hemolytic condition.

The disclosure further provides a method for treating a subject having a condition associated with elevated blood pressure in the lungs, *e.g.* pulmonary hypertension, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite. In some embodiments, this

includes treating a subject having neonatal pulmonary hypertension. In some embodiments, this includes treating a subject having primary and/or secondary pulmonary hypertension. In some embodiments for treating subjects having a condition associated with elevated blood pressure in the lungs, the nitrite is nebulized.

Also contemplated herein are methods for treating, ameliorating, or preventing other conditions of or associated with blood flow, including vasospasm, stroke, angina, revascularization of coronary arteries and other arteries (peripheral vascular disease), transplantation (*e.g.*, of kidney, heart, lung, or liver), treatment of low blood pressure (such as that seen in shock or trauma, surgery and cardiopulmonary arrest) to prevent reperfusion injury to vital organs, cutaneous ulcers (*e.g.*, with topical, non-acidified nitrite salt), Raynauds phenomenon, treatment of hemolytic conditions (such as sickle cell, malaria, TTP, and HUS), hemolysis caused by immune incompatibility before and after birth, and other conditions listed herein.

Also provided herein are methods of administering a pharmaceutically-acceptable nitrite salt to a subject, for treating, preventing or ameliorating a condition selected from: (a) ischemia-reperfusion injury (*e.g.*, hepatic or cardiac or brain ischemia-reperfusion injury); (b) pulmonary hypertension (*e.g.*, neonatal pulmonary hypertension); or (c) cerebral artery vasospasm. Also contemplated are methods for treatment, prevention, and/or amelioration of gestational or fetal cardiovascular malconditions.

The foregoing and other features and advantages will become more apparent from the following detailed description of several embodiments, which proceeds with reference to the accompanying figures.

Brief Description of the Figures

Figure 1 is a graph, depicting hemodynamic and metabolic measurements at baseline and during exercise in 18 subjects. **Figure 1A** shows effects on each of the indicated values without inhibition of NO synthesis. **Figure 1B** shows effects with inhibition of NO synthesis. *Key*: MAP – mean arterial pressure, mmHg; FBF – forearm blood flow, mL/min/100mL; O_2 saturation, %; pO_2 – venous oxyhemoglobin saturation, partial pressure of oxygen, mmHg; pH, units; * = $p<0.05$ vs. Baseline 1 or 2, respectively; ** = $p<0.01$ vs. Baseline 1 or 2, respectively; † = $p<0.05$ vs. Baseline 1; †† = $p<0.01$ vs. Initial Exercise.

Figure 2 is a graph, depicting effects of infusion of sodium nitrite in bicarbonate-buffered normal saline into the brachial arteries of 18 healthy subjects. **Figure 2A** shows effects on each of the indicated values without inhibition of NO synthesis. **Figure 2B** shows effects with inhibition of NO synthesis. *Key as for Figure 1, plus*: Nitrite – venous nitrite, μM; NO-heme – venous iron-nitrosyl-hemoglobin, μM; and MetHb – venous methemoglobin, %; + = $p<0.01$ vs. Initial Exercise.

Figure 3 is a series of graphs, illustrating the effects of infusion of low-dose sodium nitrite into the brachial arteries of 10 healthy subjects at baseline and during exercise, without and with inhibition of NO synthesis. **Figure 3A** shows forearm blood flow at baseline and following a five-

minute infusion of $NaNO_2$. **Figure 3B** shows forearm blood flow with and without low-dose nitrite infusion at baseline and during L-NMMA infusion with and without exercise stress. **Figure 3C** shows venous levels of nitrite from the forearm circulation at the time of blood flow measurements. **Figure 3D** shows venous levels of S-nitroso-hemoglobin (S-NO) and iron-nitrosyl-hemoglobin (Hb-NO) at baseline and following nitrite infusion during exercise stress.

Figure 4 is a pair of graphs, showing formation of NO-hemoglobin adducts. **Figure 4A** shows formation of iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin, comparing baseline, with nitrite infusion, and nitrite infusion with exercise. **Figure 4B** compares formation of NO-hemoglobin adducts with hemoglobin-oxygen saturation in the human circulation, during nitrite infusion.

Figure 5A shows NO release following nitrite injections into solutions of PBS ("PBS"), deoxygenated red blood cells ("deoxy-RBC"), and oxygenated red blood cells ("oxy-RBC"). **Figure 5B** shows the rate of NO formation from nitrite mixed with PBS (first bar in each set), and oxygenated and deoxygenated red blood cells (second and third bar in each set, respectively).

Figure 6 is a mutlipanel figure showing nitrite therapy in hepatic ischemia-reperfusion injury. **Figure 6A** illustrates the experimental protocol used for murine model of hepatic ischemia-reperfusion injury. **Figure 6B** is a graph showing serum AST levels in mice following hepatic ischemia-reperfusion. *$p < 0.05$ vs. vehicle (0 μM) and **$p < 0.01$ vs. vehicle (0 μM) **Figure 6C** is a graph showing serum ALT levels in mice following hepatic ischemia-reperfusion. *$p < 0.05$ vs. vehicle (0 μM) and **$p < 0.01$ vs. vehicle (0 μM) **Figure 6D** is a representative photomicrographs of hepatic histopathology following 45 minutes of ischemia and 24 hours of reperfusion. **Figure 6E** is a bar graph showing pathological scoring of hepatic tissue samples following 45 minutes of ischemia and 24 hours of reperfusion. **Figure 6F** is a bar graph showing hepatocellular apoptosis as measured by TUNEL staining following 45 minutes of ischemia and 24 hours of reperfusion. ** $p < 0.001$ vs. I/R alone group

Figure 7 is a multipanel figure showing nitrite therapy in myocardial ischemia-reperfusion injury. **Figure 7A** illustrates the experimental protocol used for myocardial ischemia-reperfusion studies in mice. **Figure 7B** is a representative photomicrographs of the murine hearts following 30 minutes of myocardial ischemia and reperfusion. **Figure 7C** is a bar graph comparing myocardial area-at-risk (AAR) per left ventricle (LV), infarct size (INF) per AAR, and infarct per left ventricle in mice treated with nitrate or nitrite. **Figure 7D** is a bar graph comparing myocardial ejection fraction at baseline and following 45 minutes of myocardial ischemia and 48 hours of reperfusion. **Figure 7E** is a bar graph comparing left ventricular fractional shortening at baseline and following 45 minutes of myocardial ischemia and 48 hours of reperfusion.

Figure 8 is a series of graphs, illustrating blood and liver tissue levels of nitrite, RSNO and RxNO. **Figure 8A** shows blood nitrite, RSNO, and RxNO levels (μmol/L) in animals (n=3-5 per group) subjected to sham hepatic ischemia-reperfusion (I/R) or hepatic ischemia and either 1 or 30 minutes of reperfusion. ** $p < 0.001$ vs. sham **Figure 8B** shows liver tissue nitrite levels in mice (n=3-5 per group) subjected to hepatic ischemia-reperfusion (I/R) injury. **Figure 8C** shows liver tissue RSNO levels (μmol/L) in mice (n = 3-5 per group) subjected to hepatic ischemia and varying

periods of reperfusion. **Figure 8D** shows hepatic tissue RxNO levels (μmol/L) following hepatic ischemia and reperfusion in mice (n = 3-5 per group).

Figure 9 is a multipanel figure, illustrating nitrite mediated hepatoprotection and the nitric oxide and heme oxygenase-1 signaling pathways. **Figure 9A** is a graph, comparing serum aspartate aminotransferase (AST) levels in mice receiving saline vehicle, nitrite (24 μM), the nitric oxide (NO) scavenger PTIO, or nitrite (24 μM) + PTIO. **p < 0.01 vs. the vehicle group. **Figure 9B** is a graph comparing serum levels of AST in eNOS deficient (-/-) mice receiving saline vehicle or sodium nitrite (24 μM). **Figure 9C** is an image showing hepatic protein levels of heme oxygenase-1 (HO-1) determined using western blot analysis in sham operated animals and in animals subjected to hepatic ischemia (45 minutes) and reperfusion (5 hours). **Figure 9D** is a graph comparing serum AST levels in mice treated with nitrite (24 μM) or the HO-1 inhibitor zinc deuteroporphyrin bis glycol (ZnDPBG) in the setting of hepatic ischemia reperfusion injury.

Figure 10 is a series of panels, showing the effects of nitrite anion inhalation in newborn hypoxic lambs (*n*=7) **(Figure 10A)** on hemodynamic and metabolic measurements. After a hypoxic gas mixture (FiO_2 =0.12) had been started at time 0, nitrite by aerosol reduced pulmonary artery pressure (PAP) from hypoxic levels by 63 +/- 3% (*P* <0.01 versus hypoxic baseline) with little change in mean arterial pressure (MAP), cardiac output, or methemoglobin levels, but a marked increase in exhaled NO (*P* <0.01 compared to baseline). **Figure 10B** illustrates the effect of saline inhalation on pulmonary artery pressure in hypoxic lambs (*n*=7). **Figure 10C** is a multipanel graph, showing maximal effects of nitrite nebulization as compared to saline nebulization on PAP, MAP, and exhaled NO (eNO). Data are mean ± SEM.

Figure 11 illustrates effects of nitrite anion inhalation in newborn lambs during stable, normoxic (SaO_2 ~ 99 %) pulmonary hypertension induced by the infusion of an endoperoxide analog of thromboxane (U46619) (*n*=6). After infusion of U46619 was started at time 0, nitrite by aerosol reduced pulmonary artery pressure (PAP) from infusion baseline level by 23 ± 6% (*P* <0.05 compared to infusion baseline) with no measurable change in mean arterial pressure (MAP) and with a moderate increase in exhaled NO (*P* <0.01 compared to baseline).

Figure 12A compares the change in pulmonary arterial pressure (PAP), exhaled NO, and iron-nitrosyl-hemoglobin as measured by both chemiluminescence and electron paramagnetic resonance (EPR) after nitrite inhalation in animals with pulmonary hypertension induced with either hypoxia or infusion of the thromboxane analog U46199. Data for iron-nitrosyl-hemoglobin, measured by areas of output peaks after tri-iodide based reductive chemiluminescence **(Figure 12B)** and by depth of peak at 3350 Gauss in electron paramagnetic resonance (EPR) **(Figure 12C**; red line: drug induced, blue line: hypoxic) measured 20 minutes after nitrite inhalation was begun. **Figure 12D** shows change in mean pulmonary artery pressure during hypoxia after inhalation of nebulized sodium nitrite was related to blood pH, with increased vasodilation associated with decreasing pH (*r* = 0.57 *P* = 0.055). Data are mean ± SEM.

Figure 13 is a multipanel figure, showing duration of effect of NO gas inhalation (*n*=7) **(Figure 13A)** or nitrite nebulization (*n*=7) **(Figure 13B)** on hemodynamic and metabolic

measurements during hypoxic-induced pulmonary hypertension. Treatment with nitrite aerosol resulted in a rapid sustained reduction in hypoxic-induced pulmonary vasoconstriction and a graded increase in exhaled NO gas concentration with no change in mean arterial blood pressure. These results are contrasted to the rapid return in pulmonary artery pressure to hypoxic baseline after termination of inhaled NO gas (**Figure 13A**). Methemoglobin (Met Hb) concentrations increased from 2.1 ± 0.1 % during baseline to 2.8 ± 0.2% after nitrite nebulization (P <0.05). Note that the exhaled nitric oxide concentrations in **Figure 13A** reach the limit of detection during administration of inhaled nitric oxide (20 ppm). **Figure 13C** shows the change in pulmonary artery pressure (PAP) after aerosolization of nebulized nitrite and during the remaining hour of hypoxia following the termination of nitrite nebulization. **Figure 13D** shows the arterial plasma nitrite concentrations during the course of the experiment. **Figure 13E** shows the relationship between pulmonary artery pressure and exhaled NO after nitrite nebulization during hypoxia. Data are mean ± SEM.

Figure 14 is a multi-column (panel) figure depicting experiment design, biochemical and clinical results in a series of non-human primates that received intravenous nitrite to examine its effects on the development of vasospasm of the cerebral arteries and resulting ischemia. Each of the three columns represents a separate experimental group (control, low nitrite, and high nitrite). This figure describes experimental design (upper row: arrows pointing down marking the events; small arrows pointing up in the middle column representing daily boluses of nitrite), biochemical results (linear graphs: red, nitrite levels in blood; blue, nitrite levels in CSF; green, levels of nitrosylated protein/albumin in CSF; the brown bar graph represents the methemoglobin levels in blood), and mean blood pressure (the last grey bar graph) in samples collected during the experiment.

Figure 15 presents characteristic cerebral arteriograms before SAH (Day 0 (preinfusion); **Figure 15A, 15C**) and on day 7 after SAH (**Figure 15B, 15D**) in two animals: one control treated with intravenous infusion of saline at 2 μl/min for 14 days (**Figure 15A, 15B**) and one treated with intravenous nitrite at 870 μmol/min for 14 days (**Figure 15C, 15D**). In **Figure 15B**, the arrows point to the right middle cerebral artery (R MCA) in spasm. R ICA, the right internal carotid artery, R ACA, the right anterior cerebral artery.

Figure 16 depicts degree of vasospasm of the right middle cerebral artery (R MCA) in each animal from all experimental groups (8 control, 3 low dose, and 3 high dose of nitrite). R MCA vasospasm was assessed as the area of the proximal 14-mm segment of the right MCA by three blinded examiners using a computerized image analysis system (NIH Image 6.21). Arteriographic vasospasm was quantified relative to each animal baseline arteriogram. The mean values for saline vs. nitrite groups are represented by the circles; bars represent standard deviations. Statistical significance p<0.001.

Detailed Description of the Disclosure

I. Abbreviations

ANOVA analysis of variance

carboxy-PTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt
DCV	delayed cerebral vasospasm
deoxy-RBC	deoxygenated red blood cells
eNOS	endothelial NO synthase
FiO_2	fractional concentration of inspired oxygen
FBF	forearm blood flow
iNO	inhaled nitric oxide
I/R	ischemia-reperfusion
LCA	main coronary artery
L-NMMA	L-NG-monomethyl-arginine
LV	left ventricle
NO	nitric oxide
NOS	nitric oxide synthase
MAP	mean arterial pressure
MetHb	methemoglobin
oxy-RBC	oxygenated red blood cells
PBS	phosphate buffered saline
pO_2 (or Po_2)	partial oxygen pressure
SAH	subarachnoid hemorrhage
S-NO	S-nitroso-hemoglobin

II. Terms

Unless otherwise noted, terms used herein should be accorded their standard definitions and conventional usage. For example, one of skill in the art can obtain definitions for the terms used herein in dictionaries and reference textbooks, for example: *Stedman's Medical Dictionary* (26th Ed., Williams and Wilkins, Editor M. Spraycar, 1995); *The New Oxford American Dictionary* (Oxford University Press, Eds E. Jewell and F. Abate, 2001); *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 3rd Ed., Cold Spring Harbor Laboratory Press, 2001); and *Hawley's Condensed Chemical Dictionary*, 11th Ed. (Eds. N. I. Sax and R. J. Lewis, Sr., Van Nostrand Reinhold, New York, New York, 1987); *Molecular Biology and Biotechnology: a Comprehensive Desk Reference* (VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8)).

In order to facilitate review of the various embodiments, the following explanations of specific terms are provided:

Animal: Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals.

Cerebral ischemia or ischemic stroke: A condition that occurs when an artery to or in the brain is partially or completely blocked such that the oxygen demand of the tissue exceeds the oxygen supplied. Deprived of oxygen and other nutrients following an ischemic stroke, the brain suffers damage as a result of the stroke.

Ischemic stroke can be caused by several different kinds of diseases. The most common problem is narrowing of the arteries in the neck or head. This is most often caused by atherosclerosis, or gradual cholesterol deposition. If the arteries become too narrow, blood cells may collect in them and form blood clots (thrombi). These blood clots can block the artery where they are formed (thrombosis), or can dislodge and become trapped in arteries closer to the brain (embolism).

Another cause of stroke is blood clots in the heart, which can occur as a result of irregular heartbeat (for example, atrial fibrillation), heart attack, or abnormalities of the heart valves. While these are the most common causes of ischemic stroke, there are many other possible causes. Examples include use of street drugs, traumatic injury to the blood vessels of the neck, or disorders of blood clotting.

Ischemic stroke is by far the most common kind of stroke, accounting for about 80% of all strokes. Stroke can affect people of all ages, including children. Many people with ischemic strokes are older (60 or more years old), and the risk of stroke increases with older ages. At each age, stroke is more common in men than women, and it is more common among African-Americans than white Americans. Many people with stroke have other problems or conditions which put them at higher risk for stroke, such as high blood pressure (hypertension), heart disease, smoking, or diabetes.

Fetal: A term describing the time period in the latter part of pregnancy when organ systems are functional and blood flow patterns are established for central critical organs, such as the heart, brain and lungs.

Hypoxia: Deficiency in the amount of oxygen reaching body tissues.

Injectable composition: A pharmaceutically acceptable fluid composition comprising at least one active ingredient, for example, a salt of nitrite. The active ingredient is usually dissolved or suspended in a physiologically acceptable carrier, and the composition can additionally comprise minor amounts of one or more non-toxic auxiliary substances, such as emulsifying agents, preservatives, pH buffering agents and the like. Such injectable compositions that are useful for use with the compositions of this disclosure are conventional; appropriate formulations are well known in the art.

Ischemia: A vascular phenomenon in which a decrease in the blood supply to a bodily organ, tissue, or part is caused, for instance, by constriction or obstruction of one or more blood vessels. Ischemia sometimes results from vasoconstriction or thrombosis or embolism. Ischemia can lead to direct ischemic injury, tissue damage due to cell death caused by reduced oxygen supply.

Ischemia/reperfusion injury: In addition to the immediate injury that occurs during deprivation of blood flow, ischemic/reperfusion injury involves tissue injury that occurs after blood flow is restored. Current understanding is that much of this injury is caused by chemical products and free radicals released into the ischemic tissues.

When a tissue is subjected to ischemia, a sequence of chemical events is initiated that may ultimately lead to cellular dysfunction and necrosis. If ischemia is ended by the restoration of blood flow, a second series of injurious events ensue producing additional injury. Thus, whenever there is a transient decrease or interruption of blood flow in a subject, the resultant injury involves two components - the direct injury occurring during the ischemic interval and the indirect or reperfusion injury that follows. When there is a long duration of ischemia, the direct ischemic damage, resulting from hypoxia, is predominant. For relatively short duration ischemia, the indirect or reperfusion mediated damage becomes increasingly important. In some instances, the injury produced by

reperfusion can be more severe than the injury induced by ischemia *per se*. This pattern of relative contribution of injury from direct and indirect mechanisms has been shown to occur in all organs.

Methemoglobin: The oxidized form of hemoglobin in which the iron in the heme component has been oxidized from the ferrous (+2) to the ferric (+3) state. This renders the hemoglobin molecule incapable of effectively transporting and releasing oxygen to the tissues. Normally, there is about 1% of total hemoglobin in the methemoglobin form.

Methemoglobinemia: A condition in which a substantial portion of the hemoglobin in the blood of a subject is in the form of methemoglobin, making it unable to carry oxygen effectively to the tissues. Methemoglobinemia can be an inherited disorder, but it also can be acquired through exposure to chemicals such as nitrates (nitrate-contaminated water), aniline dyes, and potassium chlorate. It is not the presence of methemoglobin but the amount that is important in the clinical setting. The following provides rough indications of symptoms associated with different levels of methemoglobin in the blood: < 1.7%, normal; 10-20%, mild cyanosis (substantially asymptomatic, though it can result in "chocolate brown" blood); 30-40%, headache, fatigue, tachycardia, weakness, dizziness; >35%, symptoms of hypoxia, such as dyspnea and lethargy; 50-60%, acidosis, arrhythmias, coma, convulsions, bradycardia, severe hypoxia, seizures; >70% usually results in death.

Neonate: A term describing the human or animal organism in the time period after birth and extending until the adjustments from fetal to newborn life are completed.

Nitrite: The inorganic anion $^{-}NO_2$ or a salt of nitrous acid (NO_2^-). Nitrites are often highly soluble, and can be oxidized to form nitrates or reduced to form nitric oxide or ammonia. Nitrite may form salts with alkali metals, such as sodium ($NaNO_2$, also known as nitrous acid sodium salt), potassium and lithium, with alkali earth metals, such as calcium, magnesium and barium, with organic bases, such as amine bases, for example, dicyclohexylamine, pyridine, arginine, lysine and the like. Other nitrite salts may be formed from a variety of organic and inorganic bases. In particular embodiments, the nitrite is a salt of an anionic nitrite delivered with a cation, which cation is selected from sodium, potassium, and arginine. Many nitrite salts are commercially available, and/or readily produced using conventional techniques.

Parenteral: Administered outside of the intestine, for example, not via the alimentary tract. Generally, parenteral formulations are those that will be administered through any possible mode except ingestion. This term especially refers to injections, whether administered intravenously, intrathecally, intramuscularly, intraperitoneally, or subcutaneously, and various surface applications including intranasal, intradermal, and topical application, for instance.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers useful in this disclosure are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the compounds herein disclosed.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced

salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Peripheral Vascular Disease (PVD): A condition in which the arteries that carry blood to the arms or legs become narrowed or occluded. This interferes with the normal flow of blood, sometimes causing pain but often causing no readily detectable symptoms at all.

The most common cause of PVD is atherosclerosis, a gradual process in which cholesterol and scar tissue build up, forming plaques that occlude the blood vessels. In some cases, PVD may be caused by blood clots that lodge in the arteries and restrict blood flow. PVD affects about one in 20 people over the age of 50, or 8 million people in the United States. More than half the people with PVD experience leg pain, numbness or other symptoms, but many people dismiss these signs as "a normal part of aging" and do not seek medical help. The most common symptom of PVD is painful cramping in the leg or hip, particularly when walking. This symptom, also known as "claudication," occurs when there is not enough blood flowing to the leg muscles during exercise, such that ischemia occurs. The pain typically goes away when the muscles are rested.

Other symptoms may include numbness, tingling or weakness in the leg. In severe cases, people with PVD may experience a burning or aching pain in an extremity such as the foot or toes while resting, or may develop a sore on the leg or foot that does not heal. People with PVD also may experience a cooling or color change in the skin of the legs or feet, or loss of hair on the legs. In extreme cases, untreated PVD can lead to gangrene, a serious condition that may require amputation of a leg, foot or toes. People with PVD are also at higher risk for heart disease and stroke.

A **"pharmaceutical agent"** or **"drug"** refers to a chemical compound or other composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject.

Placenta: A vascular organ that provides for metabolic exchange between mother and fetus in mammals. It delivers oxygen, water, and nutrients to the fetus from the mother's blood and secretes the hormones necessary for successful pregnancy. In addition, it carries wastes away from the fetus to be processed in the mother's body.

Preeclampsia: A disease of unknown cause in pregnant women, characterized by hypertension, abnormal blood vessels in the placenta, and protein in the urine. It often but not always occurs with gestational diabetes or in diabetics. Additional symptoms may include water retention, leading to swelling in the face, hands and feet, and greater weight gain. Also called toxemia. Preeclampsia can lead to eclampsia if not treated. The only known cure for preeclampsia is delivery of the child.

Preventing or treating a disease: "Preventing" a disease refers to inhibiting the full development of a disease. "Treatment" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified nitrite salt preparation is one in which the specified nitrite salt is more enriched than it is in its generative environment, for instance within a biochemical reaction chamber. Preferably, a preparation of a specified nitrite salt is purified such that the salt represents at least 50% of the total nitrite content of the preparation. In some embodiments, a purified preparation contains at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95% or more of the specified compound, such as a particular nitrite salt.

Reperfusion: Restoration of blood supply to tissue that is ischemic, due to decrease in blood supply. Reperfusion is a procedure for treating infarction or other ischemia, by enabling viable ischemic tissue to recover, thus limiting further necrosis. However, it is thought that reperfusion can itself further damage the ischemic tissue, causing reperfusion injury.

Subject: Living multi-cellular organisms, including vertebrate organisms, a category that includes both human and non-human mammals.

Therapeutic: A generic term that includes both diagnosis and treatment.

Therapeutically effective amount of [a vasodilator]: A quantity of compound, such as a nitrite salt, sufficient to achieve a desired effect in a subject being treated. For instance, this can be the amount necessary to treat or ameliorate relatively high blood pressure, or to measurably decrease blood pressure over a period of time, or to measurably inhibit an increase in blood pressure, in a subject.

An effective amount of a vasodilator may be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the effective amount will be dependent on the compound applied, the subject being treated, the severity and type of the affliction, and the manner of administration of the compound. For example, a therapeutically effective amount of an active ingredient can be measured as the concentration (moles per liter or molar-M) of the active ingredient (such as a pharmaceutically-acceptable salt of nitrite) in blood (*in vivo*) or a buffer (*in vitro*) that produces an effect.

By way of example, as described herein it is now shown that pharmaceutically-acceptable salts of nitrite (such as sodium nitrite) are effective as vasodilators at calculated dosages of about 0.6 to about 200 μM final concentration of nitrite in the circulating blood of a subject, which level can be determined empirically or through calculations. Specific levels can be reached, for instance, by providing less than about 200 mg or less nitrite in a single dose, or a dose provided over a period of time (*e.g.*, by infusion or inhalation). For instance, other dosages may be 150 mg, 100 mg, 75 mg, 50 mg or less. Specific example dosages of nitrite salts are provided herein, though the examples are not intended to be limiting. Exact dosage amounts will vary by the size of the subject being treated, the duration of the treatment, the mode of administration, and so forth.

Particularly beneficial therapeutically effective amounts of a vasodilator, such as a pharmaceutically-acceptable nitrite salt (*e.g.*, sodium nitrite), are those that are effective for vasodilation or increasing blood flow, but not so high that a significant or toxic level of methemoglobin is produced in the subject to which the vasodilator is administered. In specific embodiments, for instance, no more than about 25% methemoglobin is produced in the subject. More preferably, no more than 20%, no more than 15%, no more than 10%, no more than 8% or less methemoglobin is produced, for instance as little as 5% or 3% or less, in response to treatment with the vasodilator.

The compounds discussed herein have equal application in medical and veterinary settings. Therefore, the general term "subject being treated" is understood to include all animals (for example, humans, apes, laboratory animals, companion animals, etc.) that are or may be suffering from an aberration in blood pressure, such as hypertension.

Vasoconstriction. The diminution of the caliber or cross-sectional area of a blood vessel, for instance constriction of arterioles leading to decreased blood flow to a body part. This can be caused by a specific **vasoconstrictor**, an agent (for instance a chemical or biochemical compound) that causes, directly or indirectly, constriction of blood vessels. Such an agent can also be referred to as a **vasohypertonic** agent, and is said to have **vasoconstrictive** activity. A representative category of vasoconstrictors is the **vasopressor** (from the term pressor, tending to increase blood pressure), which term is generally used to refer to an agent that stimulates contraction of the muscular tissue of the capillaries and arteries.

Vasoconstriction also can be due to vasospasm, inadequate vasodilatation, thickening of the vessel wall, or the accumulation of flow-restricting materials on the internal wall surfaces or within the wall itself. Vasoconstriction is a major presumptive or proven factor in aging and in various clinical conditions including progressive generalized atherogenesis, myocardial infarction, stroke, hypertension, glaucoma, macular degeneration, migraine, hypertension and diabetes mellitus, among others.

Vasodilation. A state of increased caliber of the blood vessels, or the act of dilation of a blood vessel, for instance dilation of arterioles leading to increased blood flow to a body part. This can be caused by a specific **vasodilator**, an agent (for instance, a chemical or biochemical compound) that causes, directly or indirectly, dilation of blood vessels. Such an agent can also be referred to as a **vasohypotonic** agent, and is said to have **vasodilative** activity.

Vasospasm: Another cause of stroke occurs secondary to spasm of blood vessels supplying the brain. This type of stroke typically follows a subarchnoid aneurismal hemorrhage with a delayed development of vasospasm within 2-3 weeks of the bleeding event. A similar type of stroke may complicate sickle cell disease.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates

otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Hence "comprising A or B" means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

III. Overview of Several Embodiments

It has been surprisingly discovered that administration of pharmaceutically-acceptable salts of nitrite is useful in the regulation of the cardiovascular system. It has also been surprisingly discovered that nitrite is reduced to nitric oxide *in vivo*, and that the nitric oxide produced thereby is an effective vasodilator. These effects surprisingly occur at doses that do not produce clinically significant methemoglobinemia. These discoveries now enable methods to prevent and treat conditions associated with the cardiovascular system, for example, high blood pressure, pulmonary hypertension, cerebral vasospasm and tissue ischemia-reperfusion injury. These discoveries also provide methods to increase blood flow to tissues, for example, to tissues in regions of low oxygen tension. It is particularly surprising that the nitrite does not need to be applied in an acidified condition in order for it to be effective in regulating the cardiovascular system, and more particularly to act as a vasodilator *in vivo*.

Accordingly, the present disclosure provides in one embodiment a method for decreasing a subject's blood pressure, including administering to the subject sodium nitrite at about 36 μmoles per minute or less into the forearm brachial artery or intravenously.

The present disclosure also provides a method for decreasing a subject's blood pressure, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to decrease (or lower, or reduce) the subject's blood pressure. Another embodiment is a method for treating a subject having a condition associated with elevated blood pressure, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the elevated blood pressure. Also provided is a method for treating a subject having a hemolytic condition, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the hemolytic condition.

The present disclosure additionally provides a method for increasing blood flow to a tissue of a subject, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to increase blood flow to a tissue of the subject. Also provided is a method

for producing an amount of NO in a subject effective the decrease the subject's blood pressure, including administering a pharmaceutically-acceptable nitrite to the subject.

The present disclosure further provides a pharmaceutical composition comprising an effective amount of a pharmaceutically-acceptable nitrite and a carrier.

In some embodiments, the vascular complication is one or more selected from the group consisting of pulmonary hypertension (including neonatal pulmonary hypertension, primary pulmonary hypertension, and secondary pulmonary hypertension), systemic hypertension, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, an ischemic central nervous system event, and death.

In some embodiments, nitrite is administered to neonates to treat pulmonary hypertension.

In some embodiments, the hemolytic condition includes one or more selected from: sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, rhabdomyolysis (myoglobinemia), transfusion of aged blood, cardiopulomonary bypass, and hemodialysis.

In some embodiments, the decreased blood flow to the tissue is caused directly or indirectly by at least one of the following conditions: sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, rhabdomyolysis (myoglobinemia), transfusion of aged blood, transfusion of hemoglobin, transfusion of red blood cells, cardiopulmonary bypass, coronary disease, cardiac ischemia syndrome, angina, iatrogenic hemolysis, angioplasty, myocardial ischemia, tissue ischemia, hemolysis caused by intravascular devices, hemodialysis, pulmonary hypertension, systemic hypertension, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, and an ischemic central nervous system event.

In some embodiments, the tissue is an ischemic tissue. In some embodiments, the administration is parenteral, oral, bucal, rectal, *ex vivo*, or intraocular. In some embodiments, the administration is peritoneal, intravenous, intraarterial, subcutaneous, inhaled, or intramuscular. In

some embodiments, the nitrite is administered to the subject in an environment of low oxygen tension, or acts in an area of the subject's body that displays relatively low oxygen tension. In some embodiments, the nitrite is administered as a pharmaceutically-acceptable salt of nitrite, such as, for instance, sodium nitrite, potassium nitrite, or arginine nitrite. In some embodiments, the nitrite is administered in combination with at least one additional active agent. It is specifically contemplated that, in certain embodiments, that the subject is a mammal, for instance, a human.

The disclosure further provides a method for treating a subject having a condition associated with elevated blood pressure in the lungs, *e.g.* pulmonary hypertension, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite. In some embodiments, this includes treating a subject having neonatal pulmonary hypertension. In some embodiments, this includes treating a subject having primary and/or secondary pulmonary hypertension. In some embodiments for treating subjects having a condition associated with elevated blood pressure in the lungs, the nitrite is nebulized.

The disclosure also provides suggestions for a means of treating hypertension and/or preeclampsia in pregnant women. Such therapy would include action of nitrites on spastic and diseased blood vessels within the placenta.

The disclosure also provides suggestions for treating, *in utero*, fetuses with cardiovascular anomalies, hypertension, and/or misdirected blood flow. In such approaches, nitrite may be administered by introduction into the amniotic cavity either directly or by osmotic minipumps, the latter to achieve sustained release throughout days and weeks of pregnancy.

Thus, there is provided herein a method for inducing vasodilation and/or increasing blood flow in a subject, which method involves administering to the subject an effective amount of a pharmaceutically-acceptable salt of nitrite for a sufficient period of time to induce vasodilation and/or increase blood flow in the subject. Non-limiting examples of pharmaceutically acceptable salts of nitrite include sodium nitrite, potassium nitrite, and arginine nitrite. In examples of the provided methods, the pharmaceutically-acceptable salt of nitrite reacts in the presence of hemoglobin in the subject to release nitric oxide.

It is a specific advantage of methods provided herein that the effective amount of the pharmaceutically-acceptable salt of nitrite administered to the subject does not induce toxic levels of methemoglobin, and in many embodiments does not induced formation of clinically significant amounts of methemoglobin in the subject. Therefore, contemplated herein are methods in which the effective amount of the pharmaceutically-acceptable salt of nitrite, when administered to the subject, induces production in the subject of no more than about 25% methemoglobin; no more than about 20% methemoglobin; no more than about 10% methemoglobin; no more than about 8% methemoglobin; or no more than about 5% methemoglobin. Beneficially, examples of the provided methods induce production of even less than 5% methemoglobin, for instance no more than about 3% methemoglobin, less than 3%, less than 2%, or even less than 1%.

In one specific example of a method for inducing vasodilation and/or increasing blood flow in a subject, sodium nitrite is administered by injection at about 36 μmoles per minute for at least five minutes into the forearm brachial artery of the subject.

The effective amount of the pharmaceutically-acceptable salt of nitrite is administered, in various embodiments, to a circulating concentration in the subject of about 0.6 to 240 μM, measured locally to the site of administration or generally in the subject. It is noted that the local level of nitrite is expected to be higher than the general circulating level particularly in short delivery regimens; in long term delivery regimens, such as delivery using a pump or injector, or by inhalation, the system-wide or general nitrite level is expected to near the level measured near the administration site.

Administration of the pharmaceutically-acceptable nitrite can be, for instance, parenteral, oral, bucal, rectal, *ex vivo*, or intraocular in certain embodiments. In various embodiments, it is also contemplated that the administration of the nitrite can be peritoneal, intravenous, intraarterial, subcutaneous, inhaled, intramuscular, or into a cardiopulmonary bypass circuit. Combinations of two or more routes of administration are also contemplated.

In various embodiments of the method for inducing vasodilation and/or increasing blood flow in a subject, the subject is a mammal. It is particularly contemplated that the subject can be a human.

Combination therapy methods are contemplated, wherein the nitrite is administered in combination with at least one additional agent. By way of non-limiting examples, the additional agent is one or more selected from the list consisting of penicillin, hydroxyurea, butyrate, clotrimazole, arginine, or a phosphodiesterase inhibitor (such as sildenafil).

In another embodiment of the method for inducing vasodilation and/or increasing blood flow in a subject, the subject has elevated blood pressure, and the method is a method for treating at least one vascular complication associated with the elevated blood pressure, or the subject has a hemolytic condition, and the method is a method for treating at least one vascular complication associated with the hemolytic condition. Optionally, the subject may have both elevated blood pressure and a hemolytic condition.

In examples of the methods provided herein, the at least one vascular complication is one or more selected from the group consisting of pulmonary hypertension, systemic hypertension, peripheral vascular disease, trauma, cardiac arrest, general surgery, organ transplantation, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, angina, an ischemia-reperfusion event, an ischemic central nervous system event, and death.

In examples of the methods in which the subject has a hemolytic condition, the hemolytic condition is one or more selected from the group consisting of sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia,

secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, rhabdomyolysis (myoglobinemia), transfusion of aged blood, transfusion of hemoglobin, transfusion of red blood cells, cardiopulmonary bypass, coronary disease, cardiac ischemia syndrome, angina, iatrogenic hemolysis, angioplasty, myocardial ischemia, tissue ischemia, hemolysis caused by intravascular devices, and hemodialysis.

In yet another embodiment of the method for inducing vasodilation and/or increasing blood flow in a subject, the subject has a condition associated with decreased blood flow to a tissue, and the method is a method to increase blood flow to the tissue of the subject. For instance, in examples of this method, the decreased blood flow to the tissue is caused directly or indirectly by at least one condition selected from the group consisting of: sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, rhabdomyolysis (myoglobinemia), transfusion of aged blood, transfusion of hemoglobin, transfusion of red blood cells, cardiopulmonary bypass, coronary disease, cardiac ischemia syndrome, angina, iatrogenic hemolysis, angioplasty, myocardial ischemia, tissue ischemia, hemolysis caused by intravascular devices, hemodialysis, pulmonary hypertension, systemic hypertension, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, and an ischemic central nervous system event.

It is specifically contemplated in examples of this method that the tissue is an ischemic tissue, for instance one or more tissues selected from the group consisting of neuronal tissue, bowel tissue, intestinal tissue, limb tissue, lung tissue, central nervous tissue, or cardiac tissue.

Also provided are methods for inducing vasodilation and/or increasing blood flow in a subject having elevated blood pressure, wherein the elevated blood pressure comprises elevated blood pressure in the lungs. By way of example, it is contemplated that such subject in some instances has neonatal pulmonary hypertension, or primary and/or secondary pulmonary hypertension.

In examples of embodiments where the elevated blood pressure, or need for increased blood flow, in the subject comprises elevated blood pressure or need for increased blood flow in the lungs, the pharmaceutically-acceptable salt of nitrite is nebulized.

By way of example, in various embodiments the pharmaceutically-acceptable salt of nitrite is administered to a circulating concentration in the subject of no more than about 100 μM; no more than about 50 μM; no more than about 20 μM; no more than about 16 μM; or less than about 16 μM.

Another embodiment is a method for treating or ameliorating a condition selected from: (a) hepatic or cardiac or brain ischemia-reperfusion injury; (b) pulmonary hypertension; or (c) cerebral artery vasospasm, in a subject by decreasing blood pressure and/or increasing vasodilation in the subject, the method comprising administering sodium nitrite to the subject to decrease the blood pressure and/or increase vasodilation in the subject, thereby treating or ameliorating the condition.

In specific examples of this embodiment, the method is a method for treating or ameliorating hepatic or cardiac or brain ischemia-reperfusion injury. Optionally, the sodium nitrite is administered to the subject via injection, for instance, intravenous injection. In certain examples, the sodium nitrite is administered to a circulating concentration of about 0.6 to 240 μM.

In other specific examples of this embodiment, the method is a method for treating or ameliorating pulmonary hypertension, such as for instance neonatal pulmonary hypertension. Beneficially, in such methods the sodium nitrite can be administered to the subject by inhalation, for instance it can be nebulized. Optionally, in any of these methods, the sodium nitrite is administered at a rate of 270 μmol/minute, though other rates and circulating levels are contemplated.

Also provided in other examples of this embodiment are methods for treating or ameliorating cerebral artery vasospasm. Optionally, the sodium nitrite is administered to the subject via injection, for instance, intravenous injection. In examples of such methods, the sodium nitrite is administered at a rate of about 45 to 60 mg/kg.

In examples of the described methods, optionally the sodium nitrite can be administered in combination with at least one additional agent.

In any of the described methods, it is contemplated that the subject can be a mammal, such as for instance a human.

IV. Sodium Nitrite as an in vivo *vasodilator*

Nitrite anions are present in concentrations of about 150-1000 nM in the plasma and about 10 μM in aortic tissue. This represents the largest vascular storage pool of nitric oxide (NO), provided physiological mechanisms exist to reduce nitrite to NO. The vasodilator properties of nitrite in the human forearm and the mechanisms extant for its bioactivation have been investigated and results are reported herein. Sodium nitrite was infused at about 36 μmoles per minute into the forearm brachial artery of 18 normal volunteers, resulting in a regional nitrite concentration of about 222 μM and an immediate about 175% increase in resting forearm blood flow. Increased blood flow was observed at rest, during NO synthase inhibition and with exercise, and resulted in increased tissue perfusion, as demonstrated by increases in venous hemoglobin-oxygen saturation, partial pressure of oxygen, and pH. Systemic concentrations of nitrite increased to about 16 μM and significantly reduced mean arterial blood pressure. In an additional six subjects, the dose of nitrite was reduced about 2-logs and infused at 360 nmoles per minute, resulting in a forearm nitrite concentration of about 2 μM and an about 22% increase in blood flow.

Nitrite infusions were associated with the formation of erythrocyte iron-nitrosyl-hemoglobin, and to a lesser extent, S-nitroso-hemoglobin across the forearm vasculature. The

formation of NO-modified hemoglobin appears to result from the nitrite reductase activity of deoxyhemoglobin, linking tissue hypoxia and nitrite bioactivation.

These results indicate that physiological levels of blood and tissue nitrite represent a major bioavailable pool of NO that contributes to vaso-regulation and provides a mechanism for hypoxic vasodilation via reaction of vascular nitrite with deoxygenated heme proteins. Substantial blood flow effects of nitrite infusion into the brachial artery of normal human subjects results from forearm nitrite concentrations as low as about 0.9μM.

By way of example, as described herein it is now shown that pharmaceutically-acceptable salts of nitrite (such as sodium nitrite) are effective as vasodilators at calculated dosages of about 0.6 to about 200 μM final concentration of nitrite in the circulating blood of a subject. Specific circulating levels (locally or generally in the subject) can be reached, for instance, by providing less than about 200 mg or less nitrite in a single dose, or a dose provided over a period of time (*e.g.*, by infusion or inhalation). For instance, other dosages may be 150 mg, 100 mg, 75 mg, 50 mg or less. Specific example dosages of nitrite salts are provided herein, though the examples are not intended to be limiting. Exact dosage amounts will vary by the size of the subject being treated, the duration of the treatment, the mode of administration, and so forth.

Infusion rates can be calculated, for any given desired target circulating concentration, by using the following equation:

Infusion rate (μM/min) = target concentration (μmol/L, or μM) x Clearance (L/min)
where Clearance (L/min) = 0.015922087 x weight of the subject (kg) ^0.8354

The rate of clearance has been calculated based on empirical results, including those reported herein.

By way of example, when sodium nitrite is infused into a human forearm at 36 micromoles (μMol) per minute, the concentration measured coming out of forearm is about 222 μM and about 16 μM in whole body, after 15 minutes infusion. The background level of circulating nitrite in mammals is low, around 150-500 nanoM.

Particularly beneficial therapeutically effective amounts of a vasodilator, such as a pharmaceutically-acceptable nitrite salt (*e.g.*, sodium nitrite), are those that are effective for vasodilation or increasing blood flow, but not so high that a significant or toxic level of methemoglobin is produced in the subject to which the vasodilator is administered. In specific embodiments, for instance, no more than about 25% methemoglobin is produced in the subject. More preferably, no more than 20%, no more than 15%, no more than 10%, no more than 8% or less methemoglobin is produced, for instance as little as 5% or 3% or less, in response to treatment with the vasodilator.

By way of specific example, nitrite can be infused at concentrations less than 40 μMol per minute intravenously or intraarterially, or given by mouth. Importantly, doses used are less than those used for the treatment of cyanide poisoning, which are designed to induce clinically significant methemoglobinemia. Surprisingly, the doses described herein for the treatment/prevention of

cardiovascular conditions produce significant and beneficial clinical effects without clinically significant methemoglobin production.

Relatively complex inorganic/organic nitrite compounds and nitrate compounds have been utilized clinically to treat disorders, including angina. These drugs (*e.g.*, glyceryl trinitrate) suffer from tolerance (requiring increases in dosage in order to maintain the same effect), however, and are distinct vasodilators compared to nitrite. For example, the former require cellular thiols for metabolism, whereas nitrite or the nitrite salts discussed herein (*e.g.*, sodium nitrite) do not.

V. A mechanism of iron-nitrosyl- and S-nitroso-hemoglobin formation in vivo

The levels of both iron-nitrosyl- and S-nitroso-hemoglobin formed *in vivo* in this study are striking. During a transit time of less than 10 seconds through the forearm circulation during exercise, infused nitrite (200 μM regional concentration) produced approximately 750 nM iron-nitrosyl-hemoglobin and 200 nM SNO-Hb. The formation of both NO-hemoglobin adducts was inversely correlated with hemoglobin-oxygen saturation, which fell during exercise stress, measured from the antecubital vein by co-oximetry (for iron-nitrosyl-hemoglobin $r=-0.7$, $P<0.0001$; for S-nitroso-hemoglobin $r=-0.45$, $P=0.04$; Figure 4B). Addition of 200 μM nitrite to whole blood at different oxygen tensions (0-100%) recapitulated the *in vivo* data with increasing concentrations of iron-nitrosyl hemoglobin being formed at lower oxygen tensions (for iron-nitrosyl-hemoglobin $r=-0.968$, $P<0.0001$; for S-nitroso-hemoglobin $r=-0.45$, $P=0.07$), strongly suggesting that the NO and SNO formation was dependent on the reaction of nitrite with deoxyhemoglobin.

These data are consistent with the reaction of nitrite with deoxyhemoglobin to form NO and iron-nitrosyl-hemoglobin (Doyle *et al.*, *J Biol Chem, 256*, 12393-12398, 1981). Nitrite is first reduced to form NO and methemoglobin with a rate constant of 2.9 $M^{-1}sec^{-1}$ (measured at 25°C, pH 7.0). This reaction will be pseudo-first order, governed by the amounts (20 mM) of intra-erythrocytic hemoglobin, and limited by the rate of nitrite uptake by the erythrocyte membrane. NO then binds to deoxyhemoglobin to form iron-nitrosyl-hemoglobin, escapes the erythrocyte, or reacts with other higher oxides, such as NO_2, to form N_2O_3 and S-nitroso-hemoglobin.

Equation series 1

NO_2^- (nitrite) + $HbFe^{II}$ (deoxyhemoglobin) + $H^+ \rightarrow$ $HbFe^{III}$ (methemoglobin) + NO + OH^-

NO + $HbFe^{II}$ (deoxyhemoglobin) $\rightarrow$ $HbFe^{II}NO$ (iron-nitrosyl-hemoglobin)

The formation of significant amounts of S-nitroso-hemoglobin *in vivo* during nitrite infusion was also observed. Luschinger and colleagues (*Proc Natl Acad Sci USA, 100*, 461-6, 2003) recently proposed that nitrite reacts with deoxyhemoglobin to make iron-nitrosyl-hemoglobin, with subsequent "transfer" of the NO to the cysteine 93 to form S-nitroso-hemoglobin mediated by reoxygenation and quaternary T to R transition of hemoglobin. However, a direct transfer of NO from the heme to the thiol requires NO oxidation to NO+ and such "cycling" has not been reproduced by other research groups. Fernandez and colleagues have recently suggested that nitrite catalyzes the

reductive nitrosylation of methemoglobin by NO, a process that generates the intermediate nitrosating species dinitrogen teraoxide (N_2O_3) (*Inorg Chem, 42*, 2-4, 2003). However, nitrite reactions with hemoglobin provide ideal conditions for NO and S-nitrosothiol generation along the oxygen gradient as nitrite reacts with deoxyhemoglobin to form NO and with oxyhemoglobin to form nitrogen dioxide (NO_2) radical. NO_2 participates in radical-radical reactions ($k=10^9$ $M^{-1}sec^{-1}$) with NO to form N_2O_3 and S-nitrosothiol. Additional chemistry of nitrite with hemoglobin produces reactive oxygen metabolites (such as superoxide and hydrogen peroxide; Watanabe *et al.*, *Acta Med Okayama 35*, 173-8, 1981; Kosaka *et al.*, *Biochim Biophys Acta 702*, 237-41, 1982; and Kosaka *et al.*, *Environ Health Perspect 73*, 147-51, 1987). Chemistry involving such NO radical- oxygen radical reactions provides competitive pathways for S-nitrosothiol formation in the presence of high affinity NO sinks, such as hemoglobin.

VI. Physiological Considerations

The last decade has seen an increase in the understanding of the critical role nitric oxide (NO) plays in vascular homeostasis. The balance between production of NO and scavenging of NO determines NO bioavailability, and this balance is carefully maintained in normal physiology. The homeostatic, vasoregulatory system is apparently fine-tuned to scavenge excess NO to limit gross endocrine actions while allowing for sufficient local NO necessary for regional tonic vasodilation. However, rapid NO scavenging by cell-free hemoglobin disrupts this balance (Reiter *et al.*, *Nat Med* 8, 1383-1389, 2002). Under normal physiological conditions, hemoglobin is rapidly and effectively cleared by the hemoglobin scavenger system. However, chronic hemolytic conditions, such as sickle cell disease, result in the daily release of substantial quantities of hemoglobin into the vasculature, suggesting that cell-free hemoglobin may have major systemic effects on NO bioavailability. A current focus of research attempts to explain and treat the vascular complications common to many chronic hemolytic conditions, such as pulmonary hypertension, cutaneous ulceration and acute and chronic renal failure. Similarly, a number of clinical diseases and therapies such as acute hemolytic crises, hemolysis during cardiopulmonary bypass procedures, transfusion of aged blood, and myoglobinuria following muscle infarction are often complicated by acute pulmonary and systemic hypertension, acute renal failure, intravascular thrombosis, ischemic central nervous system events and/or death.

It is demonstrated herein that nitrite produces vasodilation in humans associated with nitrite reduction to NO by deoxyhemoglobin. Remarkably, systemic levels of 16 μM resulted in systemic vasodilation and decreased blood pressure, and regional forearm levels of only 1-2 μM significantly increased blood flow at rest and with exercise stress. Furthermore, conversion of nitrite to NO and S-nitrosothiol was mediated by reaction with deoxyhemoglobin, providing a mechanism for hypoxia-regulated catalytic NO production by the erythrocyte or endothelial/tissue heme proteins. While high concentrations of hemoglobin in red cells, coupled with the near diffusion-limited reaction rates ($\sim 10^7$ $M^{-1}s^{-1}$) of NO with hemoglobin, seem to prohibit NO from being exported from the red blood cell, the data presented herein argue to the contrary. While not intending to be limiting, perhaps unique

characteristics of the erythrocyte membrane, with a submembrane protein and methemoglobin-rich microenvironment, and the relative lipophilic nature of NO, allow compartmentalized NO production at the red blood cell membrane. This, coupled with the small yields of NO necessary for vasodilation, could account for the export of NO despite these kinetic constraints. It is further proposed that *in vivo* chemistry for the conversion of nitrite to NO and S-nitrosothiol by reaction with deoxyhemoglobin and methemoglobin provides a mechanism for hypoxia-regulated catalytic NO production by the erythrocyte or endothelial tissue heme proteins.

Three factors uniquely position nitrite, rather than S-nitrosothiol, as the major vascular storage pool of NO: 1) Nitrite is present in substantial concentrations in plasma, erythrocytes and in tissues (Rodriguez *et al., Proc Natl Acad Sci USA* 100:336-341, 2003). 2) Nitrite is relatively stable, because it is not readily reduced by intracellular reductants, as are S-nitrosothiols (Gladwin *et al., J Biol Chem* 21:21, 2002) and its reaction rate with heme proteins is 10,000 times less than that of authentic NO. 3) Nitrite is only converted to NO by reaction with deoxyhemoglobin (or presumably deoxy-myoglobin, -cytoglobin, and -neuroglobin) and its "leaving group" is the met(ferric)heme protein which will not scavenge and inactivate NO (Doyle *et al., J Biol Chem* 256:12393-12398, 1981). Therefore, this pool provides the ideal substrate for NO generation during hypoxia, providing a novel mechanism for hypoxic vasodilation.

Because a deoxyhemoglobin-nitrite reductase system would result in NO formation in deoxygenating blood, such a system links hemoglobin oxygenation status to NO generation, the principle previously ascribed to S-nitroso-hemoglobin (Jia *et al., Nature* 380:221-226, 1996). Hemoglobin possesses anionic binding cavities that retain nitrite (Gladwin *et al., J Biol Chem* 21:21, 2002) and nitrite is taken up by erythrocytes through the anion exchange protein (AE1 or Band 3) or through the membrane as nitrous acid (a pH dependent process that accelerates nitrite uptake during tissue hypoxia (Shingles *et al., J Bioenerg Biomembr* 29:611-616, 1997; May *et al., Am I Physiol Cell Physiol* 279:C1946-1954, 2000). Such nitrite would provide a steady source of NO, NO_2 and S-nitrosothiol generation that would occur preferentially in hypoxic vascular territories. Because the AE1 protein binds both deoxyhemoglobin and methemoglobin and may channel nitrite, AE1 could serve to localize catalytic NO and S-nitrosothiol generation at the erythrocyte membrane, where the relatively lipophilic NO, NO_2 and N_2O_3 could react in the vicinal lipid bilayer (Figure 5). The erythrocyte membrane is lined by an unstirred outer diffusion barrier and an inner methemoglobin rich protein matrix that might further promote such NO and NO_2 chemistry (Coin *et al., J Biol Chem* 254:1178-1190, 1979; Liu *et al., J Biol Chem* 273:18709-18713, 1998; Han *et al., Proc Natl Acad Sci USA* 99:7763-7768, 2002).

This model is consistent with the *in vitro* observations of Pawloski and colleagues (Pawloski *et al., Nature* 409:622-626, 2001) showing that S-nitrosation of hemoglobin and AE1 occurs in the erythrocyte membrane after treatment of deoxygenated red blood cells with NO solutions (which contain significant-more than 50 μM- contaminating nitrite; Fernandez,*et al.* Inorg Chem 42:2-4, 2003). Further, N_2O_3 generated at the membrane could directly nitrosate the abundant intra-erythrocytic glutathione, eliminating the requirement of transnitrosation reactions with S-nitroso-

hemoglobin and thus facilitating rapid export of low molecular weight S-nitrosothiol by simple diffusion across the erythrocyte membrane (Figure 5). A nitrite-hemoglobin chemistry supports a role for the red cell in oxygen-dependent NO homeostasis and provides a mechanism for the observations of multiple research groups that red blood cells and plasma "loaded" with NO, by exposure to NO in high concentration in solution or to NO gas or donors (in equilibria with high concentrations of nitrite), can export NO and induce vasodilation *in vitro* and *in vivo* (Rassaf *et al., J Clin Invest* 109:1241-1248, 2002; Fox-Robichaud *et al., J Glitz Invest* 101:2497-2505, 1998; McMahon *et al., Nat Med* 3:3, 2002; Cannon *et al., J Clin Invest* 108:279-287, 2001; Gladwin *et al., J Biol Chem* 21:21, 2002; Gladwin *et al., Circulation* 107:271-278, 2003; Schechter *et al., N Engl J Med* 348:1483-1485, 2003).

In addition to the reaction of nitrite with deoxyhemoglobin, reactions with deoxy-myoglobin, -cytoglobin and -neuroglobin or with other endothelial cell heme proteins may also be important. Such chemistry would occur between tissue nitrite and deoxy-myoglobin in vascular and skeletal muscle, thus contributing to hypoxic vasodilation and hypoxic potentiation of NO donors. The P_{50} of these globin monomers is approximately 3-5 mm Hg, placing their equilibrium deoxygenation point in the range of tissue pO_2 (0-10 mm Hg) during metabolic stress, such as exercise. Such a low oxygen tension reduces oxygen availability as substrate for NO synthesis, however, the tissue nitrite stores could then be reduced to NO and 5-nitrosothiol, thus sustaining critical vasodilation.

VII. Methods of Use

Therapeutic application of nitrite now can be used to provide selective vasodilation in a subject, and particularly to hypoxemic and ischemic tissue in the subject, and will be useful to treat hemolytic conditions such as sickle cell disease, where free hemoglobin released during hemolysis scavenges NO and disrupts NO-dependent vascular function. Nitrite is expected to not only inhibit the ability of free hemoglobin to scavenge NO by oxidizing it to methemoglobin, but also to generate NO in tissue beds with low oxygen tension. Thus, the applied nitrite will preferentially release nitric oxide at areas of low oxygen tension, thereby providing localized vasodilation and/or increased blood flow.

Nitrites can be administered to a subject to increase blood flow to a tissue of the subject, for example, to increase blood flow to a tissue, for instance a tissue with low oxygen tension; to cause vasodilation; to decrease a subject's blood pressure; to treat a subject having a condition associated with elevated blood pressure; to treat a hemolytic condition; to treat vascular complications associated with treatments or conditions that cause hemolysis; to treat pulmonary hypertension, cerebral vasospasm, or low blood flow to organs (such as ischemia reperfusion injury to organs including brain, heart, kidney, placenta, and liver); and/or to treat organs before and after transplantation.

Nitrite has vasodilatory properties *in vivo*

The vasodilator properties of nitrite and the mechanisms for its bioactivation were investigated as described herein. Sodium nitrite infused at 36 μmoles per minute into the forearm brachial artery of 18 normal volunteers resulted in a regional nitrite concentration of 222 μM and, surprisingly, a 175% increase in resting forearm blood flow. Increased blood flow was observed at rest, during NO synthase inhibition and with exercise. The nitrite infusion also surprisingly resulted in increased tissue perfusion, as demonstrated by increases in venous hemoglobin-oxygen saturation, partial pressure of oxygen, and pH. Increased systemic concentrations of nitrite (16 μM) significantly reduced mean arterial blood pressure.

In an additional ten subjects, the dose of nitrite was reduced 2-logs, resulting in a forearm nitrite concentration of 2 μM at rest and 0.9 μM during exercise (Figure 3). These concentrations of nitrite surprisingly significantly increased blood flow at rest and during NO synthase inhibition, with and without exercise.

Nitrite infusions were associated with the rapid formation of erythrocyte iron-nitrosyl-hemoglobin, and to a lesser extent, S-nitroso-hemoglobin across the forearm vasculature. Formation of these NO-Hb adducts was inversely proportional to the oxyhemoglobin saturation. Additionally, vasodilation of rat aortic rings and the formation of both NO gas and NO-modified hemoglobin from the nitrite reductase activity of deoxyhemoglobin and deoxygenated erythrocytes was observed, a result that links tissue hypoxia, hemoglobin allostery, and nitrite bioactivation. These results indicate that physiological levels of blood and tissue nitrite are a major bioavailable pool of NO that contributes to vaso-regulation and provide a mechanism for hypoxic vasodilation via reaction of vascular nitrite with deoxygenated heme proteins in tissue and/or the erythrocyte.

The findings described herein that administration of nitrite reduces blood pressure and increases blood flow are unexpected and surprising because published reports to date teach the person of ordinary skill in the art that pharmacological levels of nitrites (below about 100-200 μM), when administered to subjects, lack intrinsic vasodilatory properties (Lauer *et al.*, *Proc Natl Acad Sci USA,* 98:12814-9, 2001).

It is also believed that pharmaceutically acceptable salts of nitrite can be infused into patients with hemolytic disease, such as sickle cell disease, to improve blood flow, limit ischemia-reperfusion tissue injury, and oxidize cell-free plasma Hb. These effects should be useful in the treatment of sickle cell vaso-occlusive pain crisis, stroke (brain ischemia) and the acute chest syndrome.

Cytoprotective Effects of Nitrite during Ischemia-reperfusion of the Heart and Liver

The anion nitrite (NO_2^-) forms as a consequence of nitric oxide (NO) oxidation and is present at concentrations of 0.3-1.0 μM in plasma and 1-20 μM in tissue (Gladwin *et al.*, *Proc Natl Acad Sci U S A* 97:11482-11487, 2000; Rodriguez *et al.*, *Proc Natl Acad Sci U S A* 100:336-341, 2003; Rassaf *et al.*, *Nat Med* 9:481-483, 2003; Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004; Gladwin *et al.*, *J Clin Invest* 113:19-21, 2004). Nitrite has been historically considered an inert

metabolic end product with limited intrinsic biological activity (Lauer *et al.*, *Proc Natl Acad Sci U S A* 98:12814-12819, 2001; McMahon, *N Engl J Med* 349:402-405; author reply 402-405, 2003; Pawloski, *N Engl J Med* 349:402-405; author reply 402-405, 2003). Recent data from our group and others suggest that nitrite may be reduced to NO during hypoxia and acidosis (Gladwin *et al.*, *Proc Natl Acad Sci U S A* 97:11482-11487, 2000; Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004; Cosby *et al.*, *Nat Med* 9:1498-1505, 2003; Nagababu *et al.*,. *J Biol Chem* 278:46349-46356, 2003; Tiravanti *et al.*, *J Biol Chem* 279:11065-11073, 2004). At extremely low tissue pH and PO_2, nitrite may be reduced to NO by disproportionation (acidic reduction; Zweier *et al.*, *Nat Med* 1:804-809, 1995) or by the enzymatic action of xanthine oxidoreductase (Millar *et al.*, *FEBS Lett* 427:225-228, 1998; Zhang *et al.*, *Biochem Soc Trans* 25:524S, 1997; Godber *et al.*, *J Biol Chem* 275:7757-7763, 2000; Li *et al.*, *J Biol Chem* 276:24482-24489, 2001).

Nitrite represents a circulating and tissue storage form of nitric oxide (NO) whose bioactivation is mediated by the nitrite reductase activities of deoxyhemoglobin. Because the rate of NO generation from nitrite is linearly dependent on reductions in oxygen and pH, we hypothesized that nitrite would be reduced to NO in ischemic tissue and exert NO-dependent protective effects. Solutions of sodium nitrite were administered in the setting of hepatic and cardiac ischemia-reperfusion (I/R) injury in mice. In hepatic I/R, nitrite exerted profound dose dependent protective effects on cellular necrosis and apoptosis with highly significant protective effects observed at near-physiological nitrite concentrations (0.6 μM). In myocardial I/R injury, nitrite reduced cardiac infarct size by 67% and significantly improved post-ischemic left ventricular ejection fraction. Consistent with hypoxia dependent nitrite bioactivation, nitrite was reduced to NO, S-nitrosothiols, N-nitrosamines and iron-nitrosylated heme proteins within 1-30 minutes of reperfusion. Nitrite-mediated protection was dependent on NO generation and independent of eNOS and HO-1. These results suggest that nitrite is a biological storage reserve of NO subserving a critical function in tissue protection from ischemic injury. These studies evince an unexpected and novel therapy for diseases such as myocardial infarction, organ preservation and transplantation, and shock states.

Although reperfusion of ischemic tissues provides oxygen and metabolic substrates necessary for the recovery and survival of reversibly injured cells, reperfusion itself actually results in the acceleration of cellular necrosis (Braunwald *et al.*, *J. Clin. Invest.* 76:1713-1719, 1985). Ischemia-reperfusion is characterized by the formation of oxygen radicals upon reintroduction of molecular oxygen to ischemic tissues resulting in widespread lipid and protein oxidative modifications of cellular proteins, mitochondrial injury, and tissue apoptosis and necrosis (McCord *et al.*, *Adv Myocardiol* 5:183-189, 1985). In addition, following reperfusion of ischemic tissues blood flow may not return uniformly to all portions of the ischemic tissues, a phenomenon that has been termed the "no-reflow" phenomenon (Kloner *et al.*, *J Clin Invest* 54:1496-1508, 1974). Reductions in blood flow following reperfusion are thought to contribute to cellular injury and necrosis (Kloner *et al.*, *J Clin Invest* 54:1496-1508, 1974). The sudden re-introduction of blood into ischemic tissue also results in a dramatic increase in calcium delivery to the previously ischemic tissue (*i.e.*, "calcium paradox") resulting in massive tissue disruption, enzyme release, reductions in high energy phosphate

stores, mitochondrial injury, and necrosis (Nayler, *Amer. J. Path.* 102:262, 1981; Shen *et al.*, *Amer. J. Path* 67:417-440, 1972). Recent studies have also indicated that the ischemia-reperfusion injury is also characterized by an inappropriate inflammatory response in the microcirculation resulting in leukocyte-endothelial cell interactions that are mediated by the upregulation of both leukocyte and endothelial cell adhesion molecules (Lefer *et al.*, *Cardiovasc Res* 32:743-751, 1996; Entman *et al.*, *Faseb J* 5:2529-2537, 1991). Intensive research efforts have been focused on ameliorating various pathophysiological components of ischemia-reperfusion injury to limit the extent of tissue injury and necrosis.

NO, NO donors, and NO synthase activation or transgenic over-expression have been shown to exert protective effects on this process in a number of models (Lefer *et al.*, *New Horiz* 3:105-112, 1995; Lefer *et al.*, *Circulation* 88:2337-2350, 1993; Nakanishi *et al.*, *Am J Physiol* 263:H1650-1658, 1992; Jones *et al.*, *Am J Physiol Heart Circ Physiol* 286:H276-282, 2004; Jones *et al.*, *Proc Natl Acad Sci U S A* 100:4891-4896. 2003; Kanno *et al.*, *Circulation* 101:2742-2748, 2000), but in other models appears harmful (Flogel *et al.*, *J Mol Cell Cardiol* 31:827-836. 1999; Menezes *et al.*, *Am J Physiol* 277:G144-151, 1999; Woolfson *et al.*, *Circulation* 91:1545-1551, 1995; Schulz, R. *et al.*, *Cardiovasc Res* 30:432-439, 1995). Evaluation of these studies suggests a critical effect of dose and duration of NO exposure, resulting in a narrow therapeutic safety window for NO in ischemia-reperfusion pathophysiology (Bolli, *J. Mol. Cell. Cardio.* 33:1897-1918, 2001; Wink *et al.*, *Am J Physiol Heart Circ Physiol* 285:H2264-2276, 2003). An additional limitation is that NO formation from NO synthase requires oxygen as substrate, a molecule whose availability becomes limited during ischemia.

We therefore considered the use of nitrite in this context for the following reasons: (1) It is a naturally occurring substance with no potentially toxic "leaving group" (2), it is selectively reduced to NO in tissues with low oxygen tension and low pH (Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004; Cosby *et al.*, *Nat Med* 9:1498-1505, 2003; Nagababu *et al.*, *J Biol Chem* 278:46349-46356, 2003; Tiravanti *et al.*, *J Biol Chem* 279:11065-11073, 2004; Doyle *et al.*, *J Biol Chem* 256:12393-12398, 1981; Luchsinger *et al.*, *Proc Natl Acad Sci US A* 100:461-466, 2003), (3) its activation does not require molecular oxygen (Cosby *et al.*, *Nat Med* 9:1498-1505, 2003), and (4) NO is known to maintain heme proteins in a reduced and liganded state (Herold *et al.*, *Free Radic Biol Med* 34:531-545, 2003; Herold *et al.*, *J Biol Inorg Chem* 6:543-555, 2001; Fernandez *et al.*, *Inorg Chem* 42:2-4, 2003), limit free iron and heme mediated oxidative chemistry (Kanner *et al.*, *Arch Biochem Biophys* 237:314-321, 1985; Kanner *et al.*, *Lipids* 20:625-628, 1985; Kanner *et al.*, *Lipids* 27:46-49, 1992), transiently inhibit cytochome c oxidase and mitochondrial respiration (Torres *et al.*, *FEBS Lett* 475:263-266, 2000; Brown *et al.*, *FEBS Lett* 356:295-298, 1994; Cleeter *et al.*, *FEBS Lett* 345:50-54, 1994; Rakhit *et al.*, *Circulation* 103:2617-2623, 2001), and modulate apoptotic effectors (Mannick *et al.*, *Science* 284:651-654, 1999), all mechanisms that might participate in cytotoxicity following severe ischemia.

Nitric oxide has been shown to quench oxygen free radicals in a transient ischemia and reperfusion injury animal models (Mason *et al.*, *J Neurosurg* 93: 99-107, 2000), significantly limiting

volume of stroke (Pluta *et al.*, *Neurosurgery*, 48:884-892, 2001). Therefore, nitrite via releasing NO in the area of reperfusion may also have the same beneficial effect on stroke via limiting oxygen free radicals presence after reperfusion.

Furthermore, the selective opening of blood-tumor barrier by NO facilitates penetration of chemotherapeutic agents into the brain tumor (Weyerbrock *et al.*, *J. Neurosurgery*, 99:728-737, 2003); it is believed that this will also enhance penetration of other agents, particularly therapeutic agents such as radiation therapy, brain cancer. Therefore, due to hypoxic conditions within the brain tumor it is possible that nitrite can also selectively open the blood-tumor barrier providing beneficial effect in combination with chemotherapy.

Inhaled Nebulized Nitrite is a Pulmonary Vasodilator

Persistent pulmonary hypertension of the newborn occurs with an incidence of 0.43–6.8/1,000 live births and is associated with mortality rates between 10–20% (Walsh-Sukys *et al.*, *Pediatrics* 105, 14-20, 2000). Survivors may develop neurodevelopmental and audiological impairment (46%), cognitive delays (30%), hearing loss (19%) and a high rate of rehospitalization (22%) (Lipkin *et al.*, *J Pediatr* 140, 306-10, 2002).

Pulmonary hypertension occurs as a primary or idiopathic disease (Runo & Loyd, *Lancet* 361:1533-44, 2003; Trembath & Harrison, *Pediatr Res* 53:883-8, 2003), as well as secondary to a number of systemic and pulmonary diseases (Rubin, *N Engl J Med* 336:111-7, 1997). Regardless of etiology, pulmonary hypertension is associated with substantial morbidity and mortality. Newborn infants and adults with pulmonary disease often develop systemic hypoxemia, reduced oxyhemoglobin saturation and increased pulmonary vascular resistance (Rubin, *N Engl J Med* 336:111-7, 1997; Haworth, *Heart* 88:658-64, 2002). Therapeutically administered inhaled nitric oxide (NO) decreases pulmonary vascular resistance in newborns and adults and improves ventilation-to-perfusion matching and oxygenation; in newborns, inhaled NO reduces chronic lung damage and reduces the need for extracorporeal membrane oxygenation. Randomized placebo-controlled trials of inhaled NO therapy for term and near-term newborns with severe hypoxic respiratory failure demonstrated an improvement in hypoxemia and reduced need for extracorporal membrane oxygenation (Clark *et al.*, *N Engl J Med* 342, 469-74, 2000; Roberts *et al.*, *N Engl J Med* 336, 605-10, 1997; The Neonatal Inhaled Nitric Oxide Study Group. *N Engl J Med* 336, 597-604, 1997). A recent randomized placebo-controlled trial in premature infants with respiratory distress syndrome indicated that treatment with inhaled NO reduced the combined endpoint of death and chronic lung disease (Schreiber *et al.*, *N Engl J Med* 349, 2099-107, 2003).

Despite the encouraging results regarding treatment of persistent pulmonary hypertension of the newborn with inhaled NO, the therapy does have several significant limitations (Martin, *N Engl J Med* 349, 2157-9, 2003): considerable cost (Jacobs *et al.*, *Crit Care Med* 30, 2330-4, 2002; Pierce *et al.*,. *Bmj* 325, 336, 2002; Subhedar *et al.*, *Lancet* 359, 1781-2, 2002; Angus *et al.*, *Pediatrics* 112, 1351-60, 2003), technical difficulties involved in adapting NO delivery systems for neonatal transport (Kinsella *et al.*, *Pediatrics* 109, 158-61, 2002), and the lack of availability in small community

hospitals and developing countries. In addition, NO reacts with oxygen, forming the toxic nitrogen dioxide, and thus must be stored and delivered in nitrogen at high flow rates. The gas and delivery systems are costly and the requisite delivery technology is not universally available. Therefore, alternative NO-based therapies for the treatment of pulmonary hypertension are highly desirable.

The relationship between nitrite and nitric oxide has been appreciated for close to a century, with Haldane and later Hoagland recognizing that iron-nitrosylated myoglobin (NO bound to heme) formed as an end-product during nitrite-based meat curing (Gladwin, *J Clin Invest* 113, 19-21, 2004). More than fifty years ago, Furchgott and Bhadrakom reported that nitrite vasodilated aortic ring preparations *in vitro* (Furchgott & Bhadrakom, *J Pharmacol Exp Ther* 108, 129-43, 1953); this observation was later explored by Ignarro's group in experiments evaluating the role of soluble guanylyl cyclase in endothelium-dependent vasodilation (Ignarro *et al.*, *J Pharmacol Exp Ther* 218, 739-49, 1981). However, the high concentrations of nitrite, typically in the millimolar range, required to elicit vasodilation in aortic ring *in vitro* bioassays precluded consideration of nitrite as a physiological vasodilator (Lauer *et al.*, *Proc Natl Acad Sci U S A* 98, 12814-9, 2001; Pawloski, *N Engl J Med* 349, 402-5; author reply 402-5, 2003; McMahon, *N Engl J Med* 349, 402-5; author reply 402-5, 2003).

Two decades later, in human physiological studies, we observed artery-to-vein differences for nitrite across the human forearm with increased extraction occurring during NO inhalation and exercise stress with concomitant NO synthase inhibition (Gladwin *et al.*, *Proc Natl Acad Sci U S A* 97, 11482-7, 2000). This finding suggested that nitrite was being metabolized across the forearm with increased consumption during exercise. Based on these observations along with data from a number of investigators that identified mechanisms for non-enzymatic (nitrite disproportionation) (Zweier *et al.*, *Nat Med* 1, 804-9, 1995) and enzymatic (xanthine oxidoreductase) (Zweier *et al.*, *Nat Med* 1, 804-9, 1995; Millar *et al.*, *FEBS Lett* 427, 225-8, 1998; Tiravanti *et al.*, *J Biol Chem* 279:11065-11073, 2004; Li *et al.*, *J Biol Chem*, 279(17):16939-16946, 2004) reduction of nitrite to NO, we hypothesized that nitrite is reduced *in vivo* to NO in tissues under conditions of low P_{O_2} or pH. We found support for this hypothesis in studies of normal human volunteers wherein nitrite infusion into the forearm resulted in marked vasodilation even under basal conditions at near-physiological nitrite concentrations (Example 1; Cosby *et al.*, *Nat Med* 9, 1498-505, 2003). The mechanism of this vasodilation was consistent with a reaction of nitrite with deoxygenated hemoglobin to form NO, methemoglobin (Cosby *et al.*, *Nat Med* 9, 1498-505, 2003; Nagababu *et al.*, *J Biol Chem* 278, 46349-56, 2003) and other NO adducts.

This nitrite reductase activity of deoxyhemoglobin was extensively characterized by Doyle and colleagues in 1981 (Doyle *et al.*, *J Biol Chem* 256, 12393-8, 1981): nitrite appears to react with deoxyhemoglobin and a proton to form NO and methemoglobin. Such chemistry is ideally suited for hypoxic generation of NO from nitrite, as the reaction is enhanced by hemoglobin deoxygenation and acid, providing a graded production of NO from nitrite linked to physiological changes in oxygen and pH/CO_2. The observation in this current example that inhaled nitrite generates iron-nitrosyl-hemoglobin, exhaled NO gas, and produces vasodilation in proportion to decreasing levels of

oxygenation and pH further indicates that nitrite is a bioavailable storage pool of NO and that hemoglobin may have a physiological function as a nitrite reductase, potentially contributing to hypoxic vasodilation (see Example 1). In addition to these mechanistic considerations, this example supports another therapeutic application of nitrite, extending beyond its well-established role in the treatment of cyanide poisoning.

We show herein (Example 3) that this biochemical reaction can be harnessed for the treatment of neonatal pulmonary hypertension, an NO-deficient state characterized by pulmonary vasoconstriction, right-to-left shunt pathophysiology, ventilation/perfusion inhomogeneity and systemic hypoxemia. We delivered inhaled sodium nitrite by aerosol to newborn lambs with hypoxic and normoxic pulmonary hypertension. Inhaled nitrite elicited a rapid and sustained reduction (~60%) in hypoxia induced pulmonary hypertension, a magnitude approaching that of the effects of 20 ppm NO gas inhalation and which was associated with the immediate appearance of increasing levels of NO in expiratory gas. Pulmonary vasodilation elicited by aerosolized nitrite was deoxyhemoglobin- and pH-dependent and was associated with increased blood levels of hemoglobin iron-nitrosylation. Significantly, from a therapeutic standpoint, short term delivery of nitrite, dissolved in saline, via nebulization produced selective and sustained pulmonary vasodilation with no appreciable increase in blood methemoglobin levels. These data support the paradigm that nitrite is a vasodilator acting via conversion to NO, a process coupled to hemoglobin deoxygenation and protonation, and further evince a novel, simple and inexpensive potential therapy for neonatal pulmonary hypertension.

Aerosolized nitrite is an effective vasodilatory in the described newborn lamb model (Example 3). It can be readily administered by nebulization, and appears to exhibit a wide therapeutic-to-safety margin, with limited systemic hemodynamic changes and methemoglobin production. This presents an attractive therapeutic option to inhaled NO. Nitrite is an ideal "NO producing" agent in that it 1) is a naturally occurring compound in blood, alveolar lining fluid, and tissue, and 2) has no parent-compound leaving group, such as the diazenium diolates, that requires extensive toxicological study prior to translation to human disease.

Inhaled nitrite is a potent and selective vasodilator of pulmonary circulation of the newborn lamb. This further supports the paradigm that nitrite is an NO-dependent vasodilator whose bioactivation is coupled to hemoglobin deoxygenation and protonation. This has clinical applications in veterinary and medical situations, including pulmonary hypertension and other pulmonary syndromes with apparent NO deficiencies. Based on the data presented herein, it is believed that inhaled nitrite will have efficacy in all known and tested applications of inhaled NO.

Prevention of Cerebral Artery Vasospasm after Subarachnoid Hemorrhage

Further, it has been discovered that nitrite infusion can be used to prevent cerebral artery vasospasm after aneurismal hemorrhage (Example 4). Subarachnoid hemorrhage (SAH) due to the rupture of intracranial aneurysms affects 28,000 Americans annually. Almost 70% of patients with aneurysmal SAH develop severe spasm of the cerebral arteries on the seventh day after SAH.

Despite aggressive medical therapy, neurological deficits resulting from vasospasm continue to be a major cause of morbidity and mortality. Although the etiology of cerebral vasospasm is poorly understood, there is increasing evidence that erythrocyte hemolysis in the cerebrospinal fluid and decreased availability of nitric oxide (NO), a potent vasodilator, plays a significant role. Reversal of vasospasm by NO or NO prodrugs has been documented in several animal models.

Delayed cerebral vasospasm (DCV) remains the single cause of permanent neurological deficits or death in at least fifteen percent of patients following otherwise successful endovascular or surgical treatment for ruptured intracranial aneurysm. Decreased bioavailability of nitric oxide (NO) has been mechanistically associated with the development of DCV. A primate model system for cerebral artery vasospasm was used to determine whether infusions of nitrite, a naturally occurring anion that reacts with deoxyhemoglobin to form NO and S-nitrosothiol, might prevent DCV via reactions with perivascular hemoglobin.

As described in Example 4, nitrite infusions (45 mg/kg and 60 mg/kg per day) that produced blood levels of nitrite ranging from 10-60 microM with no clinically significant methemoglobin formation (<5%) were associated with increases in plasma cerebrospinal fluid nitrite and modest increases in blood methemoglobin concentrations (2% or less) without systemic hypotension, and significantly reduced the severity of vasospasm (Figures 15 and 16). No animals infused with sodium nitrite developed significant vasospasm; mean reduction in the R MCA area on day 7 after SAH was 8±9% versus 45±5%; $P < 0.001$) Pharmacological effects of nitrite infusion were associated with bioconversion of cerebrospinal fluid nitrite to S-nitrosothiol, a potent vasodilating NO donor intermediate of nitrite bioactivation. There was no clinical or pathological evidence of nitrite toxicity.

Subacute sodium nitrite infusions prevent DCV in a primate model of SAH, and do so without toxicity. These data evince a novel, safe, inexpensive, and rationally designed therapy for DCV, a disease for which no current preventative therapy exists.

The results presented herein suggest that sodium nitrite therapy may prevent tissue injury produced by metabolic products of hemoglobin, either by vascular spasm, or by other mechanisms of tissue injury by these metabolic products.

Treatment or Amelioration of Gestational or Fetal Cardiovascular Malconditions

Based on results presented herein, it is believed that nitrite, particularly pharmaceutically acceptable salts of nitrite as described herein, can be used to treat hypertension and preeclampsia during pregnancy. Such therapy would include action of nitrites on spastic and diseased blood vessels within the placenta.

Also suggested are methods for treating fetuses in utero, particularly those afflicted with cardiovascular anomalies, hypertension, and misdirected blood flow. It is believed that it may be possible to add nitrites to the amniotic fluid, and thus indirectly to the fetus, to achieve vasodilation and redistribution of blood flow before birth. By this means, fetal cardiovascular system development and function could be altered, for instance with promotion of blood flow to the brain

and heart. To be effective longer term, it is envisioned that embodiments of such fetal therapy would include the introduction of one or more mini-osmotic pumps, containing nitrite (*e.g.*, sodium nitrite), into the amniotic cavity to thereby achieve sustained, slow release. For instance, such minipumps could be used to achieve sustained release throughout days and weeks of pregnancy.

Also suggested are methods for treating fetuses in whom plasma nitrite levels may be depressed by immune incompatibility and associated hemolytic anemias. Such fetal treatment may be extended into the neonatal period. Administrated in the fetal period may include implantation of nitrite-charged osmotic minipumps into the amniotic cavity and could include aerosol inhalation after birth.

VIII. Formulations and Administration

Nitrites, including their salts, are administered to a subject in accordance to methods provided herein, in order to decrease blood pressure and/or increase vasodilation in a subject. Administration of the nitrites in accordance with the present disclosure may be in a single dose, in multiple doses, and/or in a continuous or intermittent manner, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of the nitrites may be essentially continuous over a preselected period of time or may be in a series of spaced doses. The amount administered will vary depending on various factors including, but not limited to, the condition to be treated and the weight, physical condition, health, and age of the subject. Such factors can be determined by a clinician employing animal models or other test systems that are available in the art.

To prepare the nitrites, nitrites are synthesized or otherwise obtained and purified as necessary or desired. In some embodiments of the disclosure, the nitrite is a pharmaceutically-acceptable salt of nitrite, for example, sodium nitrite. In some embodiments of the disclosure, the nitrite is not ethyl nitrite. In some embodiments of the disclosure, the sodium nitrite is not on a medical devise, for example, not on a stent. In some embodiments of the disclosure, the nitrite is not in the form of a gel. The nitrites can be adjusted to the appropriate concentration, and optionally combined with other agents. The absolute weight of a given nitrite included in a unit dose can vary. In some embodiments of the disclosure, the nitrite is administered as a salt of an anionic nitrite with a cation, for example, sodium, potassium, or arginine.

One or more suitable unit dosage forms including the nitrite can be administered by a variety of routes including topical, oral (for instance, in an enterically coated formulation), parenteral (including subcutaneous, intravenous, intramuscular and intraperitoneal), rectal, intraamnitic, dermal, transdermal, intrathoracic, intrapulmonary and intranasal (respiratory) routes.

The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods known to the pharmaceutical arts. Such methods include the step of mixing the nitrite with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the

product into the desired delivery system. By "pharmaceutically acceptable" it is meant a carrier, diluent, excipient, and/or salt that is compatible with the other ingredients of the formulation, and not deleterious or unsuitably harmful to the recipient thereof. The therapeutic compounds may also be formulated for sustained release, for example, using microencapsulation (see WO 94/ 07529, and U.S. Patent No. 4,962,091).

The nitrites may be formulated for parenteral administration (*e.g.*, by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampoules, pre-filled syringes, small volume infusion containers or in multi-dose containers. Preservatives can be added to help maintain the shelve life of the dosage form. The nitrites and other ingredients may form suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the nitrites and other ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, *e.g.*, sterile, pyrogen-free water, before use.

These formulations can contain pharmaceutically acceptable carriers and vehicles that are available in the art. It is possible, for example, to prepare solutions using one or more organic solvent(s) that is/are acceptable from the physiological standpoint, chosen, in addition to water, from solvents such as acetone, ethanol, isopropyl alcohol, glycol ethers such as the products sold under the name "Dowanol," polyglycols and polyethylene glycols, C_1-C_4 alkyl esters of short-chain acids, ethyl or isopropyl lactate, fatty acid triglycerides such as the products marketed under the name "Miglyol," isopropyl myristate, animal, mineral and vegetable oils and polysiloxanes.

It is possible to add other ingredients such as antioxidants, surfactants, preservatives, film-forming, keratolytic or comedolytic agents, perfumes, flavorings and colorings. Antioxidants such as t-butylhydroquinone, butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol and its derivatives can be added.

The pharmaceutical formulations of the present disclosure may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are available in the art. Examples of such substances include normal saline solutions such as physiologically buffered saline solutions and water. Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical formulations of the present disclosure include water and physiologically acceptable buffered saline solutions, such as phosphate buffered saline solutions. Merely by way of example, the buffered solution can be at a pH of about 6.0-8.5, for instance about 6.5-8.5, about 7-8.

The nitrites can also be administered via the respiratory tract. Thus, the present disclosure also provides aerosol pharmaceutical formulations and dosage forms for use in the methods of the disclosure. In general, such dosage forms include an amount of nitrite effective to treat or prevent the clinical symptoms of a specific condition. Any attenuation, for example a statistically significant attenuation, of one or more symptoms of a condition that has been treated pursuant to the methods of the present disclosure is considered to be a treatment of such condition and is within the scope of the disclosure.

For administration by inhalation, the composition may take the form of a dry powder, for example, a powder mix of the nitrite and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges, or, *e.g.*, gelatin or blister packs from which the powder may be administered with the aid of an inhalator, insufflator, or a metered-dose inhaler (see, for example, the pressurized metered dose inhaler (MDI) and the dry powder inhaler disclosed in Newman, S. P. in *Aerosols and the Lung*, Clarke, S. W. and Davia, D. eds., pp. 197-224, Butterworths, London, England, 1984).

Nitrites may also be administered in an aqueous solution, for example, when administered in an aerosol or inhaled form. Thus, other aerosol pharmaceutical formulations may include, for example, a physiologically acceptable buffered saline solution. Dry aerosol in the form of finely divided solid compound that is not dissolved or suspended in a liquid is also useful in the practice of the present disclosure.

For administration to the respiratory tract, for example, the upper (nasal) or lower respiratory tract, by inhalation, the nitrites can be conveniently delivered from a nebulizer or a pressurized pack or other convenient means of delivering an aerosol spray. Pressurized packs may include a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Nebulizers include, but are not limited to, those described in U.S. Patent Nos. 4,624,251; 3,703,173; 3,561,444; and 4,635,627. Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, Mass.), Schering Corp. (Kenilworth, NJ) and American Pharmoseal Co. (Valencia, CA). For intra-nasal administration, the therapeutic agent may also be administered via nose drops, a liquid spray, such as via a plastic bottle atomizer or metered-dose inhaler. Typical of atomizers are the Mistometer (Wintrop) and the Medihaler (Riker). The nitrites may also be delivered via an ultrasonic delivery system. In some embodiments of the disclosure, the nitrites may be delivered via an endotracheal tube. In some embodiments of the disclosure, the nitrites may be delivered via a face mask.

The present disclosure further pertains to a packaged pharmaceutical composition such as a kit or other container. The kit or container holds a therapeutically effective amount of a pharmaceutical composition of nitrite and instructions for using the pharmaceutical composition for treating a condition.

IX. Combination Therapies

Furthermore, the nitrite may also be used in combination with other therapeutic agents, for example, pain relievers, anti-inflammatory agents, antihistamines, and the like, whether for the conditions described or some other condition. By way of example, the additional agent is one or more selected from the list consisting of penicillin, hydroxyurea, butyrate, clotrimazole, arginine, or a phosphodiesterase inhibitor (such as sildenafil).

Generally, it is believed that therapies that have been suggested or demonstrated to be effective when combined with NO therapy, may also be effective when combined with nitrite administration. All combination therapies that have been are being studied with NO therapy (inhaled or otherwise) are likely to be worthy of study in combination with nitrite therapy. See, for instance, Uga et al., *Pediatr. Int.* 46 (1): 10-14, 2004; Gianetti *et al.*, *J Thorac. Cardiov. Sur.* 127 (1): 44-50, 2004; Stubbe et al., *Intens. Care Med.* 29 (10): 1790-1797, 2003; Wagner *et al.,* Eur. Heart J 23: 326-326 Suppl. 2002; Park *et al.*, Yonesi Med J 44 (2):219-226, 2003; Kohele, *Israel Med. Assoc. J.* 5:19-23, 2003, for discussions of combination therapies used with NO.

Furthermore, pharmaceutically-acceptable nitrite salts (such as, for instance, sodium nitrite) may be used in combinations with drugs and agents that limit the elimination rate of administered nitrites. This combination could serve to prolong the duration of action of nitrite and would include antagonists and inhibitors of enzymes affecting the elimination of nitrites or their conversion to NO.

Alternatively, the nitrite may be used in combinations with drugs and agents that augment the action of nitrites. This combination could serve to increase the strength of responses to administered nitrites.

Recombinant tissue plasminogen activator (rt-PA) and urokinase are the only drugs that have proven to open occluded brain arteries in ischemic stroke. It is believed possible that using nitrite via quenching oxygen free radicals produced in response to reperfusion may provide an additional beneficial effect.

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the invention to the particular features or embodiments described.

Example 1

Nitrite has vasodilatory properties *in vivo*

This example provides a demonstration that nitrite, administered by infusion to the forearm of human subjects, is an effective vasodilator.

Methods

Human subjects protocol.

The protocol was approved by the Institutional Review Board of the National Heart, Lung and Blood Institute, and informed consent was obtained from all volunteer subjects. Nine men and nine women, with an average age of 33 years (range 21 - 50 years), participated in the study. An additional 10 subjects returned three-six months later for a second series of experiments with low dose nitrite infusion. Volunteers had a normal hemoglobin concentration, and all were in excellent general health without risk factors for endothelial dysfunction (fasting blood sugar >120 mg/dL, low-density lipoprotein cholesterol >130 mg/dL, blood pressure >145/95 mmHg, smoking within two years, cardiovascular disease, peripheral vascular disease, coagulopathy, or any other disease

predisposing to vasculitis or Raynaud's phenomenon). Subjects with G6PD deficiency, known cytochrome B5 deficiency or a baseline methemoglobin level > 1% were excluded (no screened subjects met these exclusion criteria). Lactating and pregnant females were excluded (one subject with positive HCG levels was excluded). No volunteer subject was allowed to take any medication (oral contraceptive agents allowed), vitamin supplements, herbal preparations, nutriceuticals or other "alternative therapies" for at least one month prior to study and were not be allowed to take aspirin for one week prior to study.

Forearm blood flow measurements

Brachial artery and antecubital vein catheters were placed into the arm, with the intra-arterial catheter connected to a pressure transducer for blood pressure measurements and an infusion pump delivering normal saline at 1 mL/min. After 20 minutes of rest, baseline arterial and venous blood samples were obtained and forearm blood flow measurements were made by strain gauge venous-occlusion plethysmography, as previously reported (Panza *et al.*, *Circulation, 87*, 1468-74, 1993). A series of 7 blood flow measurements were averaged for each blood flow determination. A series of measurements termed Parts I and II were performed in randomized order to minimize a time effect on the forearm blood flow response during nitrite infusion.

Measurement of blood flow and forearm nitrite extraction during NO blockade and repetitive exercise

Part I: Following 20 minutes of 0.9% NaCl (saline) solution infusion at 1 mL/min into the brachial artery, arterial and venous blood samples were obtained for the assays described below and forearm blood flow measured. Exercise was performed by repetitive hand-grip at one-third of the predetermined maximum grip strength using a hand-grip dynamometer (Technical Products Co.) (Gladwin *et al.*, *Proc Natl Acad Sci U S A, 97*, 9943-8, 2000; Gladwin *et al.*, *Proc Natl Acad Sci U S A, 97*, 11482-11487, 2000; Cannon *et al.*, *J Clin Invest, 108*, 279-87, 2001). Each contraction lasted for 10 seconds followed by relaxation for 5 seconds. Following 5 minutes of exercise, forearm blood flow measurements were obtained during relaxation phases of exercise, and arterial and venous samples collected. Following a 20-minute rest period with continued infusion of saline into the brachial artery, repeated baseline blood samples and forearm blood flow measurements were obtained. L-NMMA was then infused at a rate of 1 mL/min (8 μmol/min) into the brachial artery. Following 5 minutes of L-NMMA infusion, forearm blood flow was measured, and arterial and venous blood samples obtained. Forearm exercise was then initiated in that arm during continued L-NMMA infusion. Forearm blood flow was measured and blood samples obtained after 5 minutes of exercise during continued L-NMMA infusion (Figure 1).

Part II: After a 30 minute rest period with continued infusion of saline, baseline measurements were obtained, the saline infusion was then stopped, and infusion of nitrite ($NaNO_2$ 36 μmol/ml in 0.9% saline) at 1 ml/min was started. Sodium nitrite for use in humans was obtained from Hope Pharmaceuticals (300 mg in 10 ml water) and 286 mg was diluted in 100 ml 0.9% saline

by the Pharmaceutical Development Service to a final concentration of 36 μmol/ml. For the final 9 subjects studied, 0.01-0.03 mM sodium bicarbonate was added to the normal saline, so as to titrate pH to 7.0-7.4. The nitrite solution was light protected and nitrite levels and free NO gas in solution measured by reductive chemiluminescence after all experiments (Gladwin *et al.*, *J Biol Chem, 21*, 21, 2002). Only 50.5 ± 40.5 nM NO was present in nitrite solutions and was unaffected by bicarbonate buffering. There was no correlation between NO levels in nitrite solutions and blood flow effects of nitrite (r = -0.23; P=0.55). After 5 minutes of nitrite infusion, forearm blood flow measurements and blood samples were obtained, with brief interruption of the nitrite infusion to obtain the arterial sample. With continued nitrite infusion, exercise was performed as described previously, with forearm blood flow measurements and blood samples obtained as described above. The nitrite infusion was stopped and saline infusion re-started during the subsequent 30-minute rest period. Following second baseline measurements, the nitrite infusion was re-initiated, along with L-NMMA at 8 μmol/min. Five minutes later, forearm blood flow measurements were performed and blood samples obtained followed by 5 minutes of exercise with continuation of nitrite and L-NMMA infusions. Final forearm blood flow measurements and blood samples obtained. At all time points during part II, blood samples were obtained from the contralateral arm antecubital vein for determination of methemoglobin and systemic levels of NO-modified hemoglobin (Figure 2, 3, and 4). The total dose of sodium nitrite infused was 36 μmol/min x 15 minutes x 2 infusions = 1.08 mmol = 75 mg (MW $NaNO_2$ = 69).

In additional studies in 10 subjects the same stages of Parts I and II protocol were followed with infusion of low dose nitrite ($NaNO_2$ 0.36 μmol/ml in 0.9% saline, infused at 1 ml/min).

Arterial and venous pH, pO_2, and pCO_2, were measured at the bedside using the i-STAT system (i-STAT Corporation, East Windsor, NJ) and methemoglobin concentration and hemoglobin oxygen saturation measured by co-oximetry.

Measurement of red blood cell S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin.

S-nitroso-hemoglobin is unstable in the reductive red blood cell environment and rapidly decays in a temperature and redox dependent fashion, independent of oxygen tension (Gladwin *et al.*, *J Biol Chem,* 21:21, 2002). To stabilize the S-nitroso-hemoglobin for measurement, the red blood cell must be rapidly oxidized with ferricyanide. Before and during nitrite infusions, blood was drawn from both the brachial artery and antecubital vein and the whole blood immediately (at the bedside to eliminate processing time) lysed 1:10 in an NO-hemoglobin "stabilization solution" of PBS containing 1% NP-40 (to solubilize membranes), 8 mM NEM (to bind free thiol and prevent artefactual S-nitrosation), 0.1 mM DTPA (to chelate trace copper), and 4 mM ferricyanide and cyanide (to stabilize S-nitrosohemoglobin and prevent artefactual ex-vivo iron-nitrosylation during processing). The samples were desalted across a 9.5 mL bed volume Sephadex G25 column to eliminate nitrite and excess reagents and partially purify hemoglobin (99% hemoglobin preparation). The hemoglobin fraction was quantified by the method of Drabkin, and hemoglobin fractions reacted with and without mercuric chloride (1:5 $HgCl_2$:heme ratio- used to differentiate S-nitrosothiol which

is mercury labile versus iron-nitrosyl which is mercury stable) and then in 0.1 M HCL/0.5%sulfanilamide (to eliminate residual nitrite; Marley *et al.*, *Free Radic Res, 32*, 1-9, 2000). The samples were then injected into a solution of tri-iodide (I_3^-) in-line with a chemiluminescent nitric oxide analyzer (Sievers, Model 280 NO analyzer, Boulder, CO). The mercury stable peak represents iron-nitrosyl-hemoglobin. This assay is sensitive and specific for both S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin to 5 nM in whole blood (0.00005% S-NO per heme) (Gladwin *et al.*, *J Biol Chem, 21*, 21, 2002).

Analysis was initially performed using red blood cell pellet, however, despite placing the sample in ice and immediately separating plasma from erythrocyte pellet, NO formed in the venous blood *ex vivo*. To measure the true *in vivo* levels, whole blood was mixed at the bedside 1:10 in the "NO-hemoglobin stabilization solution". Plasma S-nitroso-albumin formation was negligible during nitrite infusion so this bedside whole blood assay was used to limit processing time and thus more accurately characterize the *in vivo* chemistry. In a series of validation experiments, both S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin were stable in the "NO-hemoglobin stabilization solution" for 20 minutes at room temperature with no artifactual formation or decay of NO-modified species (n=6).

Chemiluminescent detection of NO gas released from deoxyhemoglobin and deoxygenated erythrocytes following nitrite addition.

To determine whether free NO radical can form from the reaction of nitrite and deoxyhemoglobin, 100 and 200 μM nitrite was mixed with 5 mL of 660 and 1000 μM deoxygenated erythrocytes in a light protected reaction vessel purged with helium or oxygen (both 21% and 100%) in-line with a chemiluminescent NO analyzer (Seivers, Boulder, CO). After allowing equilibration for 5 minutes, nitrite was injected and the rate of NO production measured. Nitrite was injected into PBS as a control and into 100 μM hemoglobin to control for the hemolysis in the 660 and 1000 μM deoxygenated erythrocyte solutions. At the end of all experiments the visible absorption spectra of the supernatant and erythrocyte reaction mixture was analyzed and hemoglobin composition deconvoluted using a least-squares algorithm. There was less than 100 μM hemolysis in the system, no hemoglobin denaturation, and significant formation of iron-nitrosyl-hemoglobin. The NO production from erythrocyte suspensions exceeded that produced from the hemolysate control, consistent with NO export from the erythrocyte.

Statistical analysis.

An *a priori* sample size calculation determined that 18 subjects would be necessary for the study to detect a 25% improvement in forearm blood flow during nitrite infusion when forearm NO synthesis had been inhibited by L-NMMA compared with normal saline infusion control values (alpha=0.05, power=0.80). Two-sided P values were calculated by paired t-test for the pair-wise comparisons between baseline and L-NMMA infusion values, between baseline and exercise values, and between nitrite and saline control values at comparable time-points of the study. Repeated

measures ANOVA were performed for artery-to-vein gradients of NO species during basal, L-NMMA infusion, and exercise conditions. Measurements shown are mean ± SEM.

Results and Discussion

Eighteen healthy subjects (9 males, 9 females; age range 21 to 50 years) were enrolled in a physiological study to determine if nitrite is a vasodilator and to examine nitrite's *in vivo* chemistry. Part I of the protocol was designed to measure the normal hemodynamic and metabolic responses to exercise and to inhibition of NO synthesis within the forearm as a control for Part II of the protocol, in which these interventions were performed during nitrite infusion. Initial baseline measurements included a mean blood pressure of 85.6 ± 3.7 mm Hg and forearm blood flow of 4.0 ± 0.3 ml/min per 100 mL tissue (Figure 1A). Repetitive hand-grip forearm exercise increased blood flow approximately 600% over resting values, and significantly decreased ipsilateral venous hemoglobin oxygen saturation, pO_2, and pH, consistent with increased oxygen consumption and CO_2 generation. Following a 20-minute rest period, repeat hemodynamic measurements showed an approximate 10% higher forearm blood flow, but no change in systemic blood pressure or forearm venous hemoglobin oxygen saturation, pO_2 and pH values compared with the initial baseline values (Figure 1B). The NO synthase inhibitor L-NMMA was then infused into the brachial artery at 8 μmol/min for 5 minutes, significantly reducing forearm blood flow by approximately 30% and significantly reducing venous hemoglobin oxygen saturation, pO_2 and pH values. Repeated forearm exercise during continued L-NMMA infusion increased blood flow, but to a significantly lower peak value compared with exercise alone ($P<0.001$). In addition, hemoglobin oxygen saturation, pO_2 and pH were significantly lower during exercise with L-NMMA than with exercise without regional NO synthase inhibition ($P<0.001$, $P<0.005$ and $P=0.027$, respectively). Mean arterial blood pressure was unchanged during all components of Part I of the protocol.

Figure 1 depicts hemodynamic and metabolic measurements at baseline and during exercise, without (Figure 1A) and with (Figure 1B) inhibition of NO synthesis in 18 subjects. Mean arterial pressure (MAP), forearm blood flow (FBF), and venous oxyhemoglobin saturation, partial pressure of oxygen (pO_2), and pH are shown for all experimental conditions. These interventions and measurements (part I of the protocol) served as a control for Part II of the protocol, in which these interventions were performed during nitrite infusion.

To determine whether nitrite has vasoactivity in humans, in Part II of the protocol sodium nitrite in bicarbonate-buffered normal saline (final concentration 36 μmol/ml) was infused into the brachial arteries of these 18 subjects to achieve an estimated intravascular concentration of approximately 200 μM (Lauer *et al., Proc Natl Acad Sci U S A, 98*, 12814-9, 2001). Following repeat baseline measurements and infusion of sodium nitrite at 1 mL/min for 5 minutes, nitrite levels in the ipsilateral antecubital vein increased from 3.32 ± 0.32 to 221.82 ± 57.59 μM (Figure 2A). Forearm blood flow increased 175% over resting values; venous hemoglobin oxygen saturation, pO_2 and pH levels significantly increased over pre-infusion values, consistent with increased perfusion of the forearm.

Systemic levels of nitrite were 16 μM as measured in the contralateral arm and were associated with a systemic effect of decreased mean blood pressure of approximately 7 mm Hg. Consistent with immediate NO generation from nitrite during an arterial-to-venous transit, iron-nitrosylated-hemoglobin in the ipsilateral antecubital vein increased from 55.7 ± 11.4 to 693.4 ± 216.9 nM during the nitrite infusion. During forearm exercise with continuation of the nitrite infusion, blood flow increased further, with evidence of metabolic stress by virtue of reduction in forearm venous hemoglobin oxygen saturation, pO_2 and pH levels from baseline values. Venous nitrite levels declined, consistent with increased blood flow to the forearm diluting the concentration of infused nitrite. Despite decreasing forearm nitrite concentrations during exercise, iron-nitrosyl-hemoglobin levels increased (Figure 2A).

Following cessation of nitrite infusion and substitution of saline as the intra-arterial infusate for 30 minutes, repeat baseline measurements showed persistent elevations in systemic levels of nitrite, iron-nitrosyl-hemoglobin and methemoglobin (Figure 2B) over values obtained prior to the infusion of nitrite almost one hour before. In addition, persistence of a vasodilator effect was also apparent, as forearm blood flow was significantly higher (4.79 ± 0.37 versus 3.94 ± 0.38 mL/min per 100 mL tissue, P=0.003) and systemic blood pressure significantly lower (82.1 ± 3.7 versus 89.2 ± 3.5 mm Hg, P=0.002) than initial pre-nitrite infusion values. During re-infusion into the brachial artery of sodium nitrite 36 μmol/ml, combined with L-NMMA 8 μmol/min in order to again inhibit regional synthesis of NO, similar vasodilator effects of nitrite on resting and exercise forearm blood flow were seen as during nitrite infusion without L-NMMA (Figure 2B). This stands in contrast to the vasoconstrictor effect of NO synthase inhibition with L-NMMA observed in Part I of the protocol (Figure 1B). Venous nitrite and iron-nitrosyl-hemoglobin levels followed similar patterns during NO inhibition as during the initial nitrite infusion.

Figure 2 depicts the effects of infusion of sodium nitrite ($NaNO_2$) in bicarbonate-buffered normal saline (0.9%; final concentration 36 μmol/ml) into the brachial arteries of 18 healthy subjects at 1 ml/min for 5 minutes at baseline and continued during exercise. Figure 2A depicts the effects without inhibition of NO synthesis. Figure 2B depicts the effects with inhibition of NO synthesis. Values for mean arterial blood pressure (MAP), forearm blood flow (FBF), venous oxyhemoglobin saturation, partial pressure of oxygen (pO_2) and pH, venous nitrite, venous iron-nitrosyl-hemoglobin and venous methemoglobin are shown for all experimental interventions.

As a test of the physiological relevance of vascular nitrite as a vasodilator, nitrite concentrations were decreased by 2-logs to 400 nmol/mL. An infusion of 1 mL/min for five minutes in 10 subjects significantly increased forearm blood flow in all ten subjects from 3.49 ± 0.24 to 4.51 ± 0.33 ml/min per 100 mL tissue (Figure 3A; P=0.0006). Blood flow significantly increased at rest and during NO synthase inhibition with and without exercise (Figure 3B; $P<0.05$ during all conditions). Mean venous nitrite levels increased from 176 ± 17 nM to 2564 ± 462 nM following a five-minute infusion and exercise venous nitrite levels decreased to 909 ± 113 nM (secondary to dilutional effects of increased flow during exercise; Figure 3C). Again, the vasodilator effects of nitrite were paralleled with an observed formation of both iron-nitrosyl-hemoglobin and S-nitroso-

hemoglobin across the forearm circulation (Figure 3D; described below). These data indicate that basal levels of nitrite, from 150-1000 nM in plasma to 10,000 nM in vascular tissue, contribute to resting vascular tone and hypoxic vasodilation.

Figure 3 depicts the effects of infusion of low-dose sodium nitrite in bicarbonate-buffered normal saline into the brachial arteries of 10 healthy subjects at baseline and during exercise, without and with inhibition of NO synthesis. Figure 3A depicts forearm blood flow at baseline and following a five-minute in fusion of $NaNO_2$ (0.36 μmol/ml in 0.9% saline, infused at 1 ml/min). Figure 3B depicts forearm blood flow with and without low-dose nitrite infusion at baseline and during L-NMMA infusion with and without exercise stress. Figure 3C depicts venous levels of nitrite from the forearm circulation at the time of blood flow measurements. Figure 3D depicts venous levels of S-nitroso-hemoglobin (S-NO) and iron-nitrosyl-hemoglobin (Hb-NO) at baseline and following nitrite infusion during exercise stress.

The vasodilatory property of nitrite during basal blood flow conditions, when tissue pO_2 and pH are not exceedingly low, was unexpected. These results indicate that the previously hypothesized mechanisms for nitrite reduction, nitrite disproportionation and xanthine oxidoreductase activity, both of which require extremely low pO_2 and pH values not typically encountered in normal physiology, are complemented *in vivo* by additional factors that serve to catalyze nitrite reduction. While ascorbic acid and other reductants, present in abundance in blood, can provide necessary electrons for nitrous acid reduction, such that the reaction might occur at physiologically attainable pH levels, it is herein reported that deoxyhemoglobin effectively reduces nitrite to NO, within one half-circulatory time. This mechanism provides a graded production of NO along the physiological oxygen gradient, tightly regulated by hemoglobin oxygen desaturation.

Intravascular formation of NO and S-nitrosothiol by reaction of nitrite with intraerythrocytic deoxyhemoglobin

Before and during nitrite infusions, blood was drawn from both the brachial artery and antecubital vein and the whole blood immediately (at the bedside to eliminate processing time) lysed 1:10 in an NO-hemoglobin "stabilization solution" and the iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin content determined by tri-iodide-based reductive chemiluminescence and electron paramagnetic resonance spectroscopy as described in Methods. The baseline levels of S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin were at the limits of detection (<50 nM or 0.0005% NO per heme) with no artery-to-vein gradients. Following nitrite infusion in Part II of the protocol venous levels of both iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin rose strikingly (Figure 4A). The formation of both NO-hemoglobin adducts occurred across the vascular bed, a half-circulatory time of less than 10 seconds. The rate of NO formation, measured as iron-nitrosyl and S-nitroso-hemoglobin and quantified by subtraction of the arterial from the venous levels with the difference being multiplied by blood flow, increased greatly during exercise, despite a significant decrease in the venous concentration of nitrite secondary to increasing blood flow diluting the

regional nitrite concentration (Figure 4A; P=0.006 for iron-nitrosyl-hemoglobin and P=0.02 for S-nitroso-hemoglobin by repeated measures ANOVA).

Figure 4A depicts formation of iron-nitrosyl-hemoglobin (black squares) and S-nitroso-hemoglobin (red circles) during nitrite infusion at baseline, during nitrite infusion and during nitrite infusion with exercise, quantified by subtraction of the arterial from the venous levels and multiplying the result by blood flow. The formation of both NO-hemoglobin adducts was inversely correlated with hemoglobin-oxygen saturation in the human circulation during nitrite infusion (for iron-nitrosyl-hemoglobin r=-0.7, p<0.0001, for S-nitroso-hemoglobin r=-0.45, p=0.04) (Figure 4B). Hemoglobin oxygen saturation was measured from the antecubital vein by co-oximetry. Asterix in all figures signify P<0.05 by paired t test or repeated measures analysis of variance.

To determine whether free NO radical can form from the reaction of nitrite and deoxyhemoglobin, 100 and 200 μM nitrite was reacted with deoxygenated erythrocytes (5 mL volume containing a total of 660 and 1000 μM in heme) in a light protected reaction vessel purged with helium in-line with a chemiluminescent NO analyzer (Seivers, Boulder, CO.). As shown in Figure 5A and 5B, the injection of nitrite into a solution of deoxygenated erythrocytes resulted in the liberation of NO into the gas phase. There was no release from nitrite in buffer control under the same conditions, and significantly less NO was released upon nitrite addition to oxygenated erythrocytes (21% and 100% oxygen). The observed rate (determined by the assessment of the area under the curve of increased steady-state NO generation following nitrite injection calculated over 120 seconds) of NO production in the 5 mL reaction volume was consistent with 47 pM NO production per second (corresponding to an estimated 300 to 500 pM NO production per second in whole blood). While NO formation rates in this experimental system may not be extrapolated to rates of NO formation *in vivo*, the experiments are consistent with two important concepts: 1) A fraction of free NO can escape auto-capture by the remaining heme groups; this is likely only possible because nitrite is only converted to NO by reaction with deoxyhemoglobin and its "leaving group" is the met(ferric)heme protein which will limit scavenging and inactivation of NO (Doyle *et al.*, *J Biol Chem, 256*, 12393-12398, 1981); and 2) The rate of NO production is increased under anaerobic conditions, consistent with a nitrite-deoxyhemoglobin reaction.

Example 2

Cytoprotective Effects of Nitrite during Ischemia-reperfusion of the Heart and Liver

As demonstrated in Example 1, nitrite is reduced to NO by reaction with deoxyhemoglobin along the physiological oxygen gradient, a chemistry whose rate is oxygen and pH dependent and that potentially contributes to hypoxic vasodilation. Based on that unexpected discovery, we proposed that hypoxia-dependent NO production from nitrite in ischemic tissue might limit ischemia-reperfusion injury. This example provides a demonstration that infusions of sodium nitrite are effective to provide cytoprotection during ischemia-reperfusion of the heart and liver.

Although reperfusion of ischemic tissues provides oxygen and metabolic substrates necessary for the recovery and survival of reversibly injured cells, reperfusion itself actually results in the acceleration of cellular necrosis (Braunwald *et al.*, *J. Clin. Invest.* 76:1713-1719, 1985). Ischemia-reperfusion is characterized by the formation of oxygen radicals upon reintroduction of molecular oxygen to ischemic tissues resulting in widespread lipid and protein oxidative modifications of cellular proteins, mitochondrial injury, and tissue apoptosis and necrosis (McCord *et al.*, *Adv Myocardiol* 5:183-189, 1985). In addition, following reperfusion of ischemic tissues blood flow may not return uniformly to all portions of the ischemic tissues, a phenomenon that has been termed the "no-reflow" phenomenon (Kloner *et al.*, *J Clin Invest* 54:1496-1508, 1974). Reductions in blood flow following reperfusion are thought to contribute to cellular injury and necrosis (Kloner *et al.*, *J Clin Invest* 54:1496-1508, 1974). The sudden re-introduction of blood into ischemic tissue also results in a dramatic increase in calcium delivery to the previously ischemic tissue (*i.e.*, "calcium paradox") resulting in massive tissue disruption, enzyme release, reductions in high energy phosphate stores, mitochondrial injury, and necrosis (Nayler, *Amer. J. Path.* 102:262, 1981; Shen *et al.*, *Amer. J. Path* 67:417-440, 1972). Recent studies have also indicated that the ischemia-reperfusion injury is also characterized by an inappropriate inflammatory response in the microcirculation resulting in leukocyte-endothelial cell interactions that are mediated by the upregulation of both leukocyte and endothelial cell adhesion molecules (Lefer *et al.*, *Cardiovasc Res* 32:743-751, 1996; Entman *et al.*, *Faseb J* 5:2529-2537, 1991). Intensive research efforts have been focused on ameliorating various pathophysiological components of ischemia-reperfusion injury to limit the extent of tissue injury and necrosis.

NO, NO donors, and NO synthase activation or transgenic over-expression have been shown to exert protective effects on this process in a number of models (Lefer *et al.*, *New Horiz* 3:105-112, 1995; Lefer *et al.*, *Circulation* 88:2337-2350, 1993; Nakanishi *et al.*, *Am J Physiol* 263:H1650-1658, 1992; Jones *et al.*, *Am J Physiol Heart Circ Physiol* 286:H276-282, 2004; Jones *et al.*, *Proc Natl Acad Sci U S A* 100:4891-4896. 2003; Kanno *et al.*, *Circulation* 101:2742-2748, 2000), but in other models appears harmful (Flogel *et al.*, *J Mol Cell Cardiol* 31:827-836. 1999; Menezes *et al.*, *Am J Physiol* 277:G144-151, 1999; Woolfson *et al.*, *Circulation* 91:1545-1551, 1995; Schulz, R. *et al.*, *Cardiovasc Res* 30:432-439, 1995). Evaluation of these studies suggests a critical effect of dose and duration of NO exposure, resulting in a narrow therapeutic safety window for NO in ischemia-reperfusion pathophysiology (Bolli, *J. Mol. Cell. Cardio.* 33:1897-1918, 2001; Wink *et al.*, *Am J Physiol Heart Circ Physiol* 285:H2264-2276, 2003). An additional limitation is that NO formation from NO synthase requires oxygen as substrate, a molecule whose availability becomes limited during ischemia.

We therefore considered the use of nitrite in this context for the following reasons:

(1) It is a naturally occurring substance with no potentially toxic "leaving group",

(2) it is selectively reduced to NO in tissues with low oxygen tension and low pH (Bryan *et al.*, *Proc Natl Acad Sci USA.*, 2004; Cosby *et al.*, *Nat Med* 9:1498-1505, 2003; Nagababu *et al.*, *J Biol Chem* 278:46349-46356, 2003; Tiravanti *et al.*, *J Biol Chem*

279:11065-11073, 2004; Doyle *et al.*, *J Biol Chem* 256:12393-12398, 1981; Luchsinger *et al.*, *Proc Natl Acad Sci USA* 100:461-466, 2003),

(3) its activation does not require molecular oxygen (Cosby *et al.*, *Nat Med* 9:1498-1505, 2003), and

(4) NO is known to maintain heme proteins in a reduced and liganded state (Herold *et al.*, *Free Radic Biol Med* 34:531-545, 2003; Herold *et al.*, *J Biol Inorg Chem* 6:543-555, 2001; Fernandez *et al.*, *Inorg Chem* 42:2-4, 2003), limit free iron and heme mediated oxidative chemistry (Kanner *et al.*, *Arch Biochem Biophys* 237:314-321, 1985; Kanner *et al.*, *Lipids* 20:625-628, 1985; Kanner *et al.*, *Lipids* 27:46-49, 1992), transiently inhibit cytochome c oxidase and mitochondrial respiration (Torres *et al.*, *FEBS Lett* 475:263-266, 2000; Brown *et al.*, *FEBS Lett* 356:295-298, 1994; Cleeter *et al.*, *FEBS Lett* 345:50-54, 1994; Rakhit *et al.*, *Circulation* 103:2617-2623, 2001), and modulate apoptotic effectors (Mannick *et al.*, *Science* 284:651-654, 1999), all mechanisms that might participate in cytotoxicity following severe ischemia.

We evaluated the effects of nitrite therapy, compared with vehicle and nitrate controls, in well characterized murine models of hepatic and myocardial ischemia-reperfusion injury. The following description provides strong evidence for a profound protective effect of nitrite on cellular necrosis and apoptosis, which is believed to be mediated by a hypoxia-dependent bioconversion of nitrite to NO and nitros(yl)ated proteins.

Materials and Methods

Chemicals and Reagents: Sodium nitrite (S-2252) and sodium nitrate (S-8170) were obtained from the Sigma Chemical Co. (St. Louis, MO). Sodium nitrite and sodium nitrate were dissolved in phosphate buffered saline and the pH was adjusted to 7.4. In all experiments a final volume of 50 μL of sodium nitrite or sodium nitrate were administered to the mice to achieve final concentrations of circulating nitrite of 0.6 to 240 μM assuming a total circulating blood volume of 2mL. Carboxy-PTIO [2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt], a direct intravascular NO scavenger, was utilized to inhibit NO dependent effects following hepatic I/R injury. Carboxy-PTIO (Alexis Biochemicals) was dissolved in phosphate buffered saline and administered intravenously at a dose of 1 mg/Kg in a volume of 50 μL at 30 minutes prior to hepatic ischemia. Zinc(II) Deuteroporphyrin IX-2,4-bisethyleneglycol (ZnDBG) (Alexis Biochemicals) , a heme oxygenase-1 inhibitor was injected i.p. at a dose of 10 mg/Kg in a volume of 50 μL at 30 minutes prior to the induction of hepatic ischemia.

Animals: All of the mice utilized in the present studies were C57BL6/J at 8-10 weeks of age obtained from the Jackson Laboratories (Bar Harbor, ME). In additional experiments of hepatic I/R injury we utilized mice completely deficient (-/-) in endothelial nitric oxide synthase (eNOS). eNOS-/- mice were originally generously donated from Dr. Paul Huang (Mass. General Hospital) and

generated in our breeding colony at LSU-Health Sciences Center. eNOS-/- mice were utilized at 8-10 weeks of age.

Hepatic Ischemia-Reperfusion (I/R) Protocol: The hepatic I/R protocol is depicted in Figure 6A and has been described previously (Hines *et al.*, *Biochem Biophys Res Commun* 284:972-976, 2001; Hines *et al.*, *Am J Physiol Gastrointest Liver Physiol* 284:G536-545, 2001). Mice were anesthetized with the combination of ketamine (100 mg/kg) and zylazine (8 mg/kg) and a midline laparotomy was performed to expose the liver. Mice were then injected with heparin (100 μg/kg, i.p.) to prevent blood clotting. The left lateral and median lobes of the liver were rendered ischemic by completely clamping the hepatic artery and the portal vein using microaneurysm clamps. This experimental model results in a segmental (70%) hepatic ischemia. This method of partial ischemia prevents mesenteric venous congestion by allowing portal decompression throughout the right and caudate lobes of the liver. The liver was then repositioned in the peritoneal cavity in its original location for 45 minutes. The liver was kept moist using gauze soaked in 0.9% normal saline. In addition, body temperature was maintained at 37°C using a heat lamp and monitoring body temperature with a rectal temperature probe. Sham surgeries were identical except that hepatic blood flow was not reduced with a microaneurysm clamp. The duration of hepatic ischemia was 45 minutes in all experiments, following which the microaneurysm clamps were removed. The duration of hepatic reperfusion was 5 hours in the studies of serum liver transaminase levels (*i.e.*, AST or ALT) and 24 hours for the studies of liver histopathology (such as hepatocellular infarction).

Liver Enzyme Determinations: Serum samples were analyzed for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using a spectrophotometric method (Sigma Chemical Co., St. Louis, MO) (Harada *et al.*, *Proc Natl Acad Sci U S A* 100:739-744, 2003). These enzymes are liver specific and are released from the liver during injury (Hines *et al.*, *Biochem Biophys Res Commun* 284:972-976, 2001; Hines *et al.*, *Am J Physiol Gastrointest Liver Physiol* 284:G536-545, 2001).

Liver Histopathology Studies: Histopathology of liver tissue was performed as previously reported (Hines *et al.*, *Biochem Biophys Res Commun* 284:972-976, 2001). Liver tissue was fixed in 10% buffered formalin for 24 hours, embedded in paraffin, and 10 μM sections stained with hematoxylin and eosin. Histopathology scoring was performed in a double blinded manner on random high power fields using the following criteria:

0- no hepatocellular damage,

1- mild injury characterized by cytoplasmic vacuolization and focal nuclear pyknosis,

2- moderate injury with dilated sinusoids, cytosolic vacuolization, and blurring of intercellular borders,

3- moderate to severe injury with coagulative necrosis, abundant sinusoidal dilation, RBC extravasation into hepatic chords, and hypereosinophilia and margination of neutrophils,

4- severe necrosis with loss of hepatic architecture, disintegration of hepatic chords, hemorrhage, and neutrophil infiltration.

Hepatocellular apoptosis was determined using the TUNEL staining kit from Roche according to the manufacturer's recommendations. Briefly, liver tissue from various treatments was fixed in buffered formalin and 10 μm sections were prepared. Sections were permeabilized on ice for 2 minutes and incubated in 50 μL TUNEL solution for 30 minutes at 37°C. Sections were then treated with 50 μL substrate solution for 10 min. and mounted under glass coverslips. The number of apoptotic nuclei was determined from 5 random 40x fields per specimen. A total of six specimens per treatment group (16 slides per group) were analyzed and compared using one-way analysis of variance with Bonferroni's post-testing.

Myocardial Ischemia-Reperfusion (I/R) Protocol: Surgical ligation of the left main coronary artery (LCA) was performed similar to methods described previously (Jones *et al.*, *Am J Physiol Heart Circ Physiol* 286:H276-282, 2004). Briefly, mice were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and pentobarbital sodium (50 mg/kg). The animals were then attached to a surgical board with their ventral side up. The mice were orally intubated with PE-90 polyethylene tubing connected to PE-240 tubing and then connected to a Model 683 rodent ventilator (Harvard Apparatus, Natick, MA). The tidal volume was set at 2.2 milliliters and the respiratory rate was set at 122 breaths per minute. The mice were supplemented with 100% oxygen via the ventilator side port. A median sternotomy was performed using an electric cautery and the proximal left main coronary artery was visualized and completely ligated with 7-0 silk suture mounted on a tapered needle (BV-1 ethicon). In the initial experiments of myocardial infarct size coronary occlusion was maintained for 30-minutes followed by removal of suture and reperfusion for 24 hours. In additional experiments of cardiac function, the proximal LCA was completely occluded for 45 minutes followed by suture removal and reperfusion for 48 hours. In these experiments, two-dimensional echocardiography was performed at baseline and again at 48 hours of reperfusion.

Myocardial Infarct Size Determination: At 24 hours of reperfusion, the mice were anesthetized as described previously, intubated, and connected to a rodent ventilator. A catheter (PE-10 tubing) was placed in the common carotid artery to allow for Evans Blue dye injection. A median sternotomy was performed and the left main coronary artery was re-ligated in the same location as before Evans Blue dye (1.2 mL of a 2.0% solution, Sigma Chemical Co.) was injected into the carotid artery catheter into the heart to delineate the ischemic zone from the nonischemic zone. The heart was rapidly excised and serially sectioned along the long axis in five, 1 mm thick sections that were then incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co.) for 5 minutes at 37°C to demarcate the viable and nonviable myocardium within the risk zone. Each of the five, 1 mm thick myocardial slices were weighed and the areas of infarction, risk, and nonischemic left ventricle were assessed by a blinded observer using computer-assisted planimetry (NIH Image 1.57). All of the procedures for the left ventricular area-at-risk and infarct size determination have been previously described (Jones *et al.*, *Am J Physiol Heart Circ Physiol* 286:H276-282, 2004).

Echocardiographic Assessment of Left Ventricular Function: Transthoracic echocardiography of the left ventricle using a 15 MHz linear array transducer (15L8) interfaced with a Sequoia C256 (Acuson) was performed in additional groups of mice (n=9 vehicle and n=10 nitrite)

subjected to 45 minutes of myocardial ischemia and 48 hours of reperfusion. Two-dimensional echocardiography was performed at baseline and at 48 hours of reperfusion as described previously (Jones *et al.*, *Am J Physiol Heart Circ Physiol* 286:H276-282, 2004; Jones *et al.*, *Proc Natl Acad Sci U S A* 100:4891-4896. 2003). Ventricular parameters were measured using leading-edge technique. M-mode (sweep speed = 200 mm/sec) echocardiograms were captured from parasternal, short and long-axis 2D views of the left ventricle (LV) at the mid-papillary level. LV percent fractional shortening (FS) was calculated according to the following equation: LV%FS = ((LVEDD-LVESD)/LVEDD) x 100. All data were calculated from 10 cardiaccycles per experiment.

HO-1 Western Blot Analysis of homogenized liver tissue samples (50 μg total protein) was performed using mouse anti-HO-1 mAb (Stressgen, Victoria, BC) at a 1:3,000 dilution and goat anti-mouse secondary Ab (Amersham Biosciences, Piscataway, NJ) at a 1:3,000 dilution.

Blood and Tissue Nitrite Determination: For blood nitrite measurements, 160 μL of whole blood was mixed with 40 μL of a nitrite stabilizing solution containing 80 mM ferricyanide, 20 mM N-ethylmaleimide (NEM), 200 μL diethylenetriaminepentaacetic acid (DTPA), and 0.2% NP-40 (concentrations provided are after mixing with whole blood). The nitrite in whole blood was then measured using tri-idodide-based reductive chemiluminescence as previously described and validated (Gladwin *et al.*, *J Biol Chem* 21:21, 2002; Yang *et al.*, *Free Radic Res* 37:1-10, 2003).

Liver tissue was homogenized using an amended protocol published by Bryan and colleagues (Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004). Harvested liver tissue was blotted dry on filter paper, weighed, and homogenized immediately in ice-cold NEM (10 mmol/L)/ DTPA (2 mmol/L) containing buffer (3:1 dilution - w/v). The buffer/tissue mix was then homogenized with a Wheaton glass-glass homogenizer. Tissue homogenates were kept on ice and analyzed within 5 minutes. The homogenate was subsequently either injected directly into triiodine to measure the sum of nitrite, mercury stable (Rx-NO) and mercury-labile (RS-NO) NO-adducts. To determine the levels of specific NO-adducts (Rx-NO and RS-NO), the sample was reacted with and without 5 mM mercuric chloride (RS-NO becomes nitrite in presence of mercuric chloride and Rx-NO is stable) and both treated with acid sulfanilamide (0.5%) to eliminate nitrite.

Statistical Analyses: Data were analyzed by two-way analysis of variance (ANOVA) with post hoc Bonferroni analysis using StatView software version 5.0 (SAS Institute, Carey, North Carolina). Data are reported as means ± standard error of the mean (SEM) with differences accepted as significant when $p < 0.05$.

Results

Intraperitoneal nitrite limits hepatic ischemia-reperfusion (I/R) injury: Intraperitoneal delivery of 1.2 - 480 nmoles of sodium nitrite (0.6 μM to 240 μM estimated final concentration in a 2 mL total blood volume of the mouse) during hepatic ischemia dose-dependently limited serum elevations of liver transaminases, aspartate amino transferase (AST) and alanine amino transferase (ALT) (Figures 6B and 6C), with a peak effect occurring at a calculated systemic concentration of 24 μM (48 nmoles added nitrite). In sharp contrast, treatment with saline or sodium nitrate (48 nmoles)

did not exert any protective effects in the setting of hepatic I/R injury. Additional studies were performed to evaluate the effects of nitrite treatment on hepatocellular injury in mice following *in vivo* hepatic ischemia (45 minutes) and more prolonged reperfusion (24 hours; Figure 6D, 6E, and 6F). The administration of nitrite at a final blood concentration of 24 μM (48 nmoles) significantly reduced hepatocellular injury at 24 hours of reperfusion compared with saline and nitrate treated animals. In addition, nitrite therapy also significantly ($p < 0.001$) attenuated the extent of hepatocellular apoptosis following 45 minutes of hepatic ischemia and 24 hours of reperfusion (Figure 6F). The extent of hepatic cell apoptosis in nitrite treated animals subjected to I/R was similar to that observed in sham operated control animals ($p = NS$).

Intraventricular Nitrite Limits Myocardial Ischemia-Reperfusion Injury: To determine whether the potent cytoprotective effects of nitrite on liver ischemia-reperfusion injury were generalizable to other organ systems, studies were next performed to evaluate the potential cardioprotective effects of acute nitrite therapy in the setting of coronary artery occlusion and reperfusion. The experimental protocol for the myocardial I/R studies is depicted in Figure 7A. Administration of nitrite (48 nmoles) into the left ventricular cavity at 5 minutes prior to reperfusion significantly ($p < 0.001$) limited myocardial infarct size (Figures 7B and 7C) compared to 48 nmoles nitrate treatment. Despite similar myocardial areas-at-risk ($p = NS$ between groups), myocardial infarct size per area-at-risk and per left ventricle were both reduced by 67% with nitrite therapy compared to nitrate.

In additional studies, mice were subjected to 45 minutes of myocardial ischemia and 48 hours of reperfusion to evaluate the effects of nitrite treatment on left ventricular performance (Figures 7D and 7E). In these studies, both myocardial ejection fraction (Figure 7D) and myocardial fractional shortening (Figure 7E) were measured using two-dimensional echocardiography at baseline and following myocardial infarction and reperfusion. Myocardial ejection fraction was similar between the vehicle and nitrite treated study groups at baseline. Following myocardial infarction and reperfusion, ejection fraction was significantly ($p < 0.001$ vs. baseline value) lower in the saline vehicle group, yet remained essentially unchanged in the nitrite treated animals ($p = NS$ vs. baseline). Additionally, ejection fraction was significantly ($p < 0.02$) greater in the nitrite group compared to the vehicle group. Similar observations were made for fractional shortening with no significant group differences at baseline. However, following myocardial infarction and reperfusion, left ventricular fractional shortening was significantly ($p < 0.001$ vs. baseline) depressed in the vehicle group, but not in the nitrite group ($p = NS$ vs. baseline) and was significantly ($p < 0.02$) greater in the nitrite group compared to the vehicle group.

Nitrite-Mediated Cytoprotection is Associated with an Acute Ischemic Reduction of Nitrite to NO and S- and N-nitrosated Proteins within the Liver: Consistent with previously described reduction of nitrite to NO and S-nitrosothiols in a reaction with deoxyhemoglobin and deoxygenated heme proteins (Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004; Cosby *et al.*, *Nat Med* 9:1498-1505, 2003; Nagababu *et al.*, *J Biol Chem* 278:46349-46356, 2003; Doyle *et al.*, *J Biol Chem* 256:12393-12398, 1981), one minute after reperfusion the levels of nitrite in the livers of saline (control) treated

mice subjected to ischemia decreased from 1.75 μM to undetectable ($p < 0.001$ vs. sham group) and levels of mercury stable NO modified proteins (likely N-nitrosamines and iron-nitrosyl proteins; RxNO) increased to approximately 750 nM (Figure 8A; $p < 0.001$). Interestingly, with nitrite treatment there was a significant ($p < 0.01$ vs. saline treated controls) increase in post-reperfusion liver levels of nitrite (Figure 8B), S-nitrosothiols (Figure 8C) and N-nitrosamines (Figure 8D) in the nitrite treated mice. These data are consistent with the thesis that nitrite is bioactivated during hypoxic stress and consistent with recent studies of Bryan and colleagues demonstrating an acute conversion of tissue nitrite to RSNO and RxNO after a systemic anoxic insult (*Proc Natl Acad Sci U S A.*, 2004). The low levels of nitrite that are cytoprotective (1.2 nmoles at lowest dose – Figure 6B and 6C) and the reductive decomposition of "native" liver nitrite in the saline treated control animals (Figure 8A) suggest that this may be a natural mechanism for hypoxic NO production and cytoprotection. Consistent with the near-physiological amounts of nitrite given, blood nitrite levels were not significantly elevated (594 ± 83 nM to 727 ± 40 nM; n=3; p=0.16) in mice treated with 48 nmoles of nitrite, the most effective dose.

Cytoprotective effects of Nitrite are NO dependent, NO synthase Independent and Heme Oxygenase Independent: Further supporting a mechanism involving the hypoxic reduction of nitrite to NO, the NO inhibitor PTIO completely inhibited protective effects of nitrite in full factorial design experiments (Figure 9A). In contrast, significant nitrite cytoprotection was observed in endothelial NO synthase (eNOS) deficient mice (Figure 9B; $p < 0.001$), suggesting that NO production from nitrite during ischemia-reperfusion is eNOS independent. While heme oxygenase 1 protein expression is significantly induced following ischemia-reperfusion in this model, and appears to confer protection (Figure 9C and 9D), in mice pre-treated with ZnDPBG (a specific and potent heme oxygenase 1 inhibitor) nitrite significantly limited tissue injury suggesting a heme oxygenase-independent effect (Figure 9C; $p < 0.05$).

Discussion

In this example, nitrite treatment significantly increased the levels of liver nitrite and nitros(yl)ated species (RSNO and RXNO), compared with saline and nitrate treated controls, and conferred a dramatic dose-dependent cytoprotective effect, limiting necrosis, apoptosis, and preserving organ function. Remarkably, the levels of nitrite added were near-physiological, with a protective effect observed at even 1.2 nmoles added nitrite (a calculated blood level of 600 nM), suggesting that this may represent an endogenous protective mechanism that buffers severe metabolic or pathophysiological stress.

Recent data suggest that nitrite concentrations vary between blood and different organs and are typically in the high nanomolar to low micromolar range. However, until recently the high concentrations required to vasodilate aortic ring preparations led to its dismissal as an important biologically active molecule. Indeed, Furchgott *et al.* (*J. Pharmaco. Exper. Thera.* 108:129-143, 1953) demonstrated in 1953 that 100 μM nitrite stimulated vasodilation of aortic ring preparations, a process later shown to be mediated by activation of soluble guanylate cyclase (Kimura *et al., J Biol*

Chem 250:8016-8022, 1975; Mittal *et al.*, *J Biol Chem* 253:1266-1271, 1978; Ignarro *et al.*, *Biochim Biophys Acta* 631:221-231, 1980; Ignarro *et al.*, *J Pharmacol Exp Ther* 218:739-749, 1981). From a physiological standpoint, the *in vivo* conversion of nitrite to NO was thought to be limited to the stomach and severely ischemic heart, where acidic reduction or disproportionation at very low pH produces gastric mucosal vasodilation (Gladwin *et al.*, *J Clin Invest* 113:19-21, 2004; Bjorne *et al.*, *J Clin Invest* 113:106-114, 2004) and apparent cardiac tissue injury and heme iron-nitrosylation (at high nitrite concentrations in ischemic *ex vivo* heart preparations; Tiravanti *et al.*, *J Biol Chem* 279:11065-11073, 2004), respectively. While xanthine oxidoreductase dependent nitrite reduction can occur at very low oxygen tensions, NO production from this system is only detectable in the presence of high concentrations of superoxide dismutase (Li *et al.*, *J Biol Chem* 279:16939-16946, 2004; Li *et al.*, *Biochemistry* 42:1150-1159, 2001).

As described in Figure 6 and Cosby *et al.* (*Nat Med* 9:1498-1505, 2003), infusions of sodium nitrite into the human circulation produced significant vasodilation at both pharmacological and near-physiological concentrations. The bioactivation of nitrite appeared to be mediated by a nitrite reductase activity of deoxygenated hemoglobin, ultimately forming NO and iron-nitrosylated hemoglobin, and to a lesser extent S-nitrosated protein species. Based on these data, a role for circulating nitrite in mediating hypoxic vasodilation was proposed, with the oxygen sensor in this case being hemoglobin (Cosby *et al.*, *Nat Med* 9:1498-1505, 2003). It is now proposed that a similar nitrite reductase activity of deoxyhemoglobin, deoxymyoglobin and/or other deoxygenated heme proteins, accounts for the formation of nitros(yl)ated proteins and apparent NO-dependent cytoprotection observed during liver and cardiac ischemia in the present example.

Though the precise mechanism of how nitrite confers tissue protection is unclear, a critical role for NO is implicated from data shown in Figure 3 and 9A. Previous studies of NO and ischemia-reperfusion have yielded conflicting reports regarding the effects of NO on the severity of I/R injury, with some studies suggesting that NO actually contributed to reperfusion injury (Woolfson *et al.*, *Circulation* 91:1545-1551, 1995; Wink *et al.*, *Am J Physiol Heart Circ Physiol* 285:H2264-2276, 2003). Our laboratory has previously demonstrated that NO donors as well as the NO precursor, L-arginine, protect against myocardial I/R injury (Lefer *et al.*, *New Horiz* 3:105-112, 1995; Nakanishi *et al.*, *Am J Physiol* 263:H1650-1658, 1992; Pabla *et al.*, *Am J Physiol* 269:H1113-1121, 1995). More recently, we demonstrated that the severity of myocardial I/R injury is markedly exacerbated in eNOS-/- mice (Jones *et al.*, *Am J Physiol* 276:H1567-1573, 1999) whereas mice with eNOS overexpression are protected against myocardial infarction and subsequent congestive heart failure (Jones *et al.*, *Am J Physiol Heart Circ Physiol* 286:H276-282, 2004; Jones *et al.*, *Proc Natl Acad Sci U S A* 100:4891-4896. 2003; Jones *et al.*, *Am J Physiol* 276:H1567-1573, 1999).

Conflicting data on the effects of NO on ischemia-reperfusion injury may be related to the dose of NO and the conditions during ischemia and reperfusion (Bolli, *J. Mol. Cell. Cardio.* 33:1897-1918, 2001). It is now well appreciated that very high, non-physiological levels of NO (*i.e.*, high micromolar and millimolar) actually promote cellular necrosis and apoptosis (Dimmeler *et al.*, *Nitric Oxide* 4:275-281, 1997), while the demonstrated cytoprotective effects of NO typically involve

nanomolar or low micromolar concentrations of NO (Lefer *et al.*, *New Horiz* 3:105-112, 1995; Lefer *et al.*, *Circulation* 88:2337-2350, 1993; Bolli, *J. Mol. Cell. Cardio.* 33:1897-1918, 2001). Additionally, studies investigating NO and NO-releasing agents under *in vitro* conditions of I/R have consistently reported deleterious effects of NO (Bolli, *J. Mol. Cell. Cardio.* 33:1897-1918, 2001), in contrast to *in vivo* studies of I/R that reported beneficial effects of NO therapy (Lefer *et al.*, *New Horiz* 3:105-112, 1995; Lefer *et al.*, *Circulation* 88:2337-2350, 1993). How NO mediates protection is also not clear, with multiple mechanisms being reported, including sGC activation, inhibition of cytochrome C oxidase and inhibition of deleterious mitochondrial calcium uptake (Torres *et al.*, *FEBS Lett* 475:263-266, 2000; Brown *et al.*, *FEBS Lett* 356:295-298, 1994; Cleeter *et al.*, *FEBS Lett* 345:50-54, 1994; Rakhit *et al.*, *Circulation* 103:2617-2623, 2001). While these data suggest that the effects of nitrite occur secondary to NO formation, the ultimate mechanism of nitrite-dependent cytoprotection is currently unknown (Luchsinger *et al.*, *Proc Natl Acad Sci US A* 100:461-466, 2003; Fernandez *et al.*, *Inorg Chem* 42:2-4, 2003; Han *et al.*, *Proc Natl Acad Sci U S A* 99:7763-7768, 2002; Crawford *et al.*, *Blood* 101:4408-4415, 2003).

An intriguing possibility is the intermediate formation of S-nitrosothiols, known to form via reactions of nitrite with deoxyhemoglobin and possibly tissue heme proteins (Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004; Cosby *et al.*, *Nat Med* 9:1498-1505, 2003; Nagababu *et al.*, *J Biol Chem* 278:46349-46356, 2003). Consistent with hypoxia dependent formation of S-nitrosothiols in red blood cells and tissues from nitrite, hepatic levels of these species were significantly higher following reperfusion (one-to-thirty minutes) in livers exposed to ischemia and nitrite. Within the relative reductive environment intracellularly, S-nitrosothiols formed via nitrite readily will be reduced to NO and activate sGC. Alternatively, S-nitrosation and subsequent effects on activity of critical proteins important in I/R induced injury and apoptotic cell death may lead to protection (Mannick *et al.*, *Science* 284:651-654, 1999).

In addition, the data reported here reveal a dynamic regulation of hepatic RxNO's, a pool of mercury stable NO-modified proteins that include N-nitrosamines and iron-nitrosyls (Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004; Gladwin *et al.*, *J Biol Chem* 21:21, 2002; Rassaf *et al.*, *Free Radic Biol Med* 33:1590-1596, 2002), during ischemia-reperfusion. In saline treated groups, RxNO levels increase at 1 minutes of reperfusion and then decrease after 30 minutes reperfusion, whereas sustained elevation in RxNO levels are observed in nitrite treated mice, suggesting that maintenance of RxNO's could be important in protecting tissues from I/R injury.

In conclusion, the data presented in this example demonstrate a remarkable function for the relatively simple inorganic anion nitrite as a potent inhibitor of liver and cardiac ischemia-reperfusion injury and infarction, as shown in a mouse model system. The effects of nitrite appear NO-dependent, with a rapid conversion of nitrite to NO and nitros(yl)ated proteins following reperfusion. Considering the known safety of nitrite as a naturally occurring anion and as an FDA approved therapeutic for cyanide poisoning, these data evince a novel, safe, and inexpensive therapy for ischemia-reperfusion injury. Such a therapy could be used to prevent or modulate organ dysfunction following, for instance, coronary and peripheral vasculature reperfusion, high risk abdominal surgery

(such as aortic aneurism repair that leads to renal acute tubular necrosis), cardiopulmonary resuscitation, and perhaps most importantly, solid organ transplantation.

Example 3

Inhaled nebulized nitrite is a hypoxia-sensitive NO-dependent selective pulmonary vasodilator

This example provides a description of use of inhaled, nebulized nitrite (specifically, sodium nitrite) to treat neonatal pulmonary hypertension.

Based on the results presented above, it is now known that the blood anion nitrite contributes to hypoxic vasodilation via a heme-based, nitric oxide (NO) generating reaction with deoxyhemoglobin and potentially other heme proteins. This biochemical reaction can be harnessed for the treatment of neonatal pulmonary hypertension, an NO-deficient state characterized by pulmonary vasoconstriction, right-to-left shunt pathophysiology, ventilation/perfusion inhomogeneity and systemic hypoxemia. As shown in this example, inhaled sodium nitrite was delivered by aerosol to newborn lambs with hypoxic and normoxic pulmonary hypertension. Inhaled nitrite elicited a rapid and sustained reduction (~60%) in hypoxia induced pulmonary hypertension, a magnitude approaching that of the effects of 20 ppm NO gas inhalation and which was associated with the immediate appearance of increasing levels of NO in expiratory gas. Pulmonary vasodilation elicited by aerosolized nitrite was deoxyhemoglobin- and pH-dependent and was associated with increased blood levels of hemoglobin iron-nitrosylation. Significantly, from a therapeutic standpoint, short term delivery of nitrite, dissolved in saline, via nebulization produced selective and sustained pulmonary vasodilation with no appreciable increase in blood methemoglobin levels. These data support the paradigm that nitrite is a vasodilator acting via conversion to NO, a process coupled to hemoglobin deoxygenation and protonation, and further evince a novel, simple and inexpensive therapy for neonatal pulmonary hypertension.

The effect of nebulized sodium nitrite versus saline, or inhaled NO, on both hypoxia-induced and drug-induced pulmonary hypertension was compared in newborn lambs. As described in this example, inhaled nitrite forms expired NO gas and circulating iron-nitrosyl-hemoglobin, and selectively vasodilates the pulmonary circulation. This vasoactivity is associated with the level of hemoglobin desaturation and blood pH in the physiologic range, supporting the physiological and therapeutic paradigm of hemoglobin as a deoxygenation-linked nitrite reductase.

Methods

Animal protocols were approved by the Institutional Animal Research Committee of Loma Linda University and were in accordance with the National Institutes of Health guidelines for use of experimental animals.

Animal preparation: Following induction of anesthesia with intravenous thiopental sodium (20 mg/Kg), the newborn lambs were orotracheally intubated and anesthesia maintained with 1% halothane until catheters were placed surgically. Thereafter halothane was discontinued and

anesthesia maintained with morphine (0.1 mg/kg/hr). After paralysis with vecuronium (0.1 mg/kg/hr) the lungs were mechanically ventilated with initial settings of pressures: 22/6 cm H_2O, frequency: 25 breaths per minute, FiO_2: 0.21, and inspiratory time: 0.6 seconds (Sechrist Model 100, Sechrist Industries, Anaheim CA, USA). Initially and throughout the normoxic experiments, ventilator settings of frequency, peak inspiratory pressure, and FiO_2 were adjusted to maintain $SaO_2 > 95\%$, PaO_2 at 90-150 Torr, and $PaCO_2$ at 35-45 Torr.

A catheter was placed in the right brachial artery to sample pre-ductal blood for gases and chemical analysis. A pediatric thermodilution catheter was passed through a femoral vein to the pulmonary artery to measure cardiac output, pulmonary artery and pulmonary capillary wedge pressure (5.0 Pediatric Swan-Ganz® thermodilution catheter, Baxter Healthcare Corporation, Irvine, CA, USA).

Catheters were placed in the femoral artery and vein for monitoring blood pressure, heart rate, and for administration of fluids and drugs. A thermocouple was placed in the femoral vein to monitor core-body temperature which was maintained at 39 C by using a warming blanket and heat lamp throughout the experiments.

After completion of the experiments, the lambs were euthanized with a proprietary euthanasia solution (Euthasol, Western Medical Supply, Arcadia, CA, USA). In selected experiments necropsy was performed to verify the position of catheters (which were correctly positioned in all cases) and to determine that the ductus arteriosus was closed (which was closed in all cases).

Hemodynamic measurements: Mean arterial pressure, mean pulmonary artery pressure, and central venous pressure were measured continuously, and pulmonary capillary wedge pressure was measured intermittently by using calibrated pressure transducers (COBE Laboratories, Lakewood, CO) zeroed at the midthoracic level. Cardiac output was measured at 15-minute intervals throughout the studies by thermodilution using a Com-2 thermodilution module (Baxter Medical, Irvine, CA, USA). Five-ml injections of ice-cold saline were used. Determinations were carried out in triplicate and results were averaged for each sampling time point. Pulmonary vascular resistance and systemic vascular resistance were calculated by using standard formulas.

Blood gas and methemoglobin analysis: Arterial and mixed venous pH, PCO_2, and PO_2 were measured in blood samples (0.3 ml) collected at intervals throughout the experiments. Blood gases were measured (ABL3, Radiometer, Copenhagen, Denmark) and oxyhemoglobin saturation and hemoglobin concentration were measured using a hemoximeter (OSM2 Hemoximeter, Radiometer, Copenhagen, Denmark). Arterial and mixed venous methemoglobin concentrations were analyzed by photometry with the OSM2 Hemoximeter using the same arterial sample as in the blood gas determinations.

Delivery of aerosolized nitrite, saline, or NO gas: Five milliliters of either aqueous sodium nitrite (1 mM solution) or saline were placed in a jet nebulizer (Hudson RCI Micro Mist Nebulizer (Hudson Respiratory Care; Temucula, CA), driven at a constant flow rate of 8 L/minute in all experiments. The sodium nitrite solution was nebulized at a rate of 270 μmol/minute. Aerosols were delivered to the inspiration loop of the ventilator. Using a jet nebulizer, it is generally thought

that <10% of a nebulized drug deposits in the lung (Coates *et al.*, *Chest* 119, 1123-30, 2001). This is the result of the dead volume of the nebulizer and the loss of drug during the expiratory phase. Lung deposition depends on particle size distribution, which is under the influence of air flow, filling volume, drug solution, and ambient temperature (Flavin *et al.*, *Pediatr Pulmonol* 2, 35-9, 1986; Suarez & Hickey, *Respir Care* 45, 652-66, 2000; Clay *et al.*, *Thorax* 38, 755-9, 1983; Clay *et al.*, *Lancet* 2, 592-4, 1983). This is a simple, inexpensive, and widely available clinical nebulizer system, though other systems could be used.

NO gas was introduced into the inspiratory limb of the breathing circuit. The inspired concentration of NO was continuously measured by chemiluminescence (CLD 700 AL, Eco Physics Inc, Ann Arbor, MI) in the inspiratory limb of the ventilator loop.

Inhalation of nitrite or saline aerosols during hypoxic- induced pulmonary vasoconstriction. Seven lambs were studied in order to demonstrate that nebulized nitrite is a selective pulmonary vasodilator in hypoxic newborn lambs. After anesthesia and instrumentation, the lambs were allowed to recover for 30 to 90 minutes while relevant hemodynamic parameters were monitored. After baseline measurements were obtained, a 30-minute period of pulmonary hypertension was induced by decreasing the FiO_2 of the inspired gas to 0.12 for 30 minutes. Ten minutes after initiation of hypoxia, either saline or sodium nitrite aerosols were administered for the remainder of the hypoxic period. After a one-hour recovery period, a second 30-minute period of hypoxia was induced again with either saline or sodium nitrite aerosols administered during the last 20 minutes. Arterial blood samples for blood gases and analytical assays were drawn and cardiac output measurements were performed at regular intervals.

Inhalation of nitrite during U46619-induced pulmonary hypertension in normoxic conditions. Six additional lambs were studied in order to evaluate the effects of nitrite nebulization on normoxic pulmonary hypertension. Stable normoxic pulmonary hypertension was induced by an infusion of a stable endoperoxide analog of thromboxane (U46619 - 9, 11-dideoxy-11α-epoxymethano-prostaglandin $F_{2\alpha}$, Cayman Chemicals, Ann Arbor, MI). The drug was dissolved in saline and was administered at a rate of 2 μg/kg/min into the femoral venous catheter for 30 minutes. Nitrite was nebulized for inhalation during the last 20 minutes of the infusion (Figure 11).

Comparison of inhaled nitrite and NO gas during hypoxic-induced pulmonary vasoconstriction: efficacy and duration of effect. This protocol was designed to compare the efficacy of nitrite with the clinical standard, 20 ppm inhaled NO gas. This concentration of NO gas is at the upper end of the therapeutic dose given to infants with primary pulmonary hypertension (Kinsella & Abman, *Semin Perinatol* 24, 387-95, 2000; Kinsella *et al.*, *Lancet* 340, 819-20, 1992), and has also been shown to be effective in reversing hypoxic vasoconstriction in newborn lambs (Frostell *et al.*, *Circulation* 83, 2038-47, 1991). A second purpose was to determine the duration of effect of a short nitrite nebulization versus NO gas inhalation on hemodynamic and physiological measurements during prolonged hypoxic-induced pulmonary vasoconstriction. After baseline measurements were performed, the lambs were made hypoxic as described above for 35 minutes.

Ten minutes after initiation of hypoxia, a 20-minute period of NO gas inhalation was initiated (20 ppm), with continuation of hypoxia for 5 minutes after cessation of NO gas delivery. Lambs were then allowed to recover for one hour. Again, after baseline measurements were made, a second 90-minute period of hypoxia was initiated. Ten minutes after initiation of hypoxia, sodium nitrite aerosol was administered for 20 minutes, with continuation of hypoxia for 60 minutes after cessation of nitrite aerosolization (Figure 13).

Measurement of exhaled NO. Exhaled NO concentration was measured with a chemiluminescence NO analyzer (NOA 280, Sievers Instruments, Inc., Boulder, CO). The chemiluminescence analyzer was calibrated with NO-free air and NO gas (45 parts per million) according to the manufacturer's recommendations. NO was sampled though a Teflon sidearm attached to a sampling port at the proximal end of the endotracheal tube through which flow passed to the analyzer at 250 ml/min.

In selected early experiments, nitrite was nebulized through a ventilator circuit with no lamb connected while NO was measured with the chemiluminescence NO analyzer. In no experiments did nitrite nebulization through the disconnected circuit result in an increase in NO concentration in the ventilated air.

Measurement of plasma nitrite and iron-nitrosyl-hemoglobin. Blood was drawn from both the brachial artery and central venous catheter and rapidly processed. Plasma was separated after centrifugation, frozen immediately on dry ice, and then stored at –70 C until assayed for nitrite using the chemiluminescence methodologies (Sievers model 280 NO-analyzer) as previously described (Cosby *et al.*, *Nat Med* 9, 1498-505, 2003; Gladwin *et al.*, *J Biol Chem* 277, 27818-28, 2002; Yang *et al.*, *Free Radic Res* 37, 1-10, 2003). The frozen red blood cell pellet was thawed, reacted in 8 mM NEM, 100 μM DTPA, and 4 mM ferricyanide, incubated for 5 minutes, and passed through a Sephadex G25 column (Yang *et al.*, *Free Radic Res* 37, 1-10, 2003; Xu *et al.*, *Proc Natl Acad Sci U S A* 100, 11303-8, 2003). The hemoglobin fraction from the G25 column was quantified by the method of Drabkin (*J. Biol. Chem.* 112, 51-65, 1935)and reacted in 0.1 M HCl/0.5%sulfanilamide to eliminate residual nitrite. The samples were then injected into a solution of tri-iodide (I_3^-) in-line with a chemiluminescent nitric oxide analyzer (Sievers, Model 280 NO analyzer, Boulder, CO). NO gas is striped in the tri-iodide solution stoichiometrically from iron-nitrosyl-hemoglobin (Yang *et al.*, *Free Radic Res* 37, 1-10, 2003).

Electron paramagnetic resonance spectroscopy of whole blood. This was carried out at 110K using a Bruker 4131VT temperature controller on an EMX 10/12 EPR spectrometer system set at 9.4 GHz, 10 mW, 5 G modulation, 0.08 s time constant, and 84 s scan time over 600 G. Each curve represents a single 84-second scan. Concentrations of iron-nitrosyl-hemoglobin were calculated by comparing the peak-to-peak heights to a standard sample.

Data acquisition and analysis. Mean arterial pressure, pulmonary artery pressure, central venous pressure, heart rate, exhaled NO concentration, and core body temperature were measured continuously. Analog signals were digitized at 100 Hz and stored using an analogue-to-digital

converter (PowerLab SP, ADInstruments, Colorado Springs, CO) and data acquisition software (Chart v 5.02 for Macintosh, ADInstruments, Colorado Springs, CO). Following the experiments, arterial blood pressure, central venous pressure, heart rate, and exhaled NO measurements were averaged into 60-second blocks.

Statistical analysis. Serial measurements of physiological variables were compared by two-way ANOVA with repeated measures with group and time as the factors. Significance of differences was evaluated with a Dunnett's post-test. Significant differences from the baseline period were evaluated using one-way-ANOVA with repeated measures with individual animals and time as the factors. Significance of differences was further evaluated with a Newman-Keul's post-test. The calculations were done using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was assumed with $P < 0.05$. Data are presented as mean ± SEM.

Results

Pulmonary vasodilatory properties of aerosolized nitrite during hypoxic-induced pulmonary vasoconstriction

In order to determine the effect of nebulized nitrite on hypoxic pulmonary hypertension, seven newborn lambs (2-10 days of age) were instrumented under general anesthesia and maintained on mechanical ventilators and morphine infusion. Following baseline stabilization, the lambs were subjected to a 30-minute period of hypoxia by lowering FiO_2 to 0.12. Nebulized nitrite or saline was administered for the last 20 minutes of the hypoxic period. Initiation of hypoxia (arterial HbO_2 ~55%) was associated with rapid increases in mean pulmonary artery pressure (from 21 ± 1 to 34 ± 2 mmHg, $P < 0.01$) (Figure 10A, 10B) and pulmonary vascular resistance (20% ($P < 0.01$)), and decreased systemic vascular resistance (~20% ($P < 0.01$)). Inhalation of nebulized nitrite but not saline (Figure 10A, 10B) resulted in a selective decrease in pulmonary artery pressure by ~60% ($P < 0.01$) (Figure 10A, 10C) and reduced pulmonary artery resistance by ~70% ($P < 0.05$) but had no measurable effect on mean arterial blood pressure (Figure 10A, 10C) or systemic vascular resistance when compared to control animals. The decrease in pulmonary artery pressure with nitrite nebulization was associated with a progressive increase in exhaled NO from 3 ± 1 to 15 ± 4 ppb (Figure 10A, 10C). Cardiac output, arterial oxyhemoglobin saturation, and methemoglobin levels did not change measurably after nitrite inhalation as compared to values during the preceding ten minutes of hypoxia (Figure 10A). Arterial Po_2 could not change appreciably in our system as this was experimentally clamped.

Pulmonary vasodilating properties of aerosolized nitrite during normoxic drug-induced pulmonary vasoconstriction

In order to contrast the effects of nebulized nitrite on pulmonary artery pressure in the presence of normal deoxyhemoglobin with those in the presence of reduced oxygenated hemoglobin, the effects of nebulized nitrite were studied in a separate group of six lambs subjected to pulmonary hypertension under normoxic conditions. Stable normoxic (SaO_2 ~98%) pulmonary hypertension

was induced by infusion of the endoperoxide analog of thromboxane (U46619). Intravenous infusion of U46619 at a rate of 2 μg/kg/min for 30 minutes was associated with rapid increases in pulmonary artery pressure from 24 ± 1 to 51 ± 4 mmHg ($P < 0.001$) (Figure 11). Ten minutes after the infusion began, addition of inhalation of nebulized nitrite resulted in a selective decrease in pulmonary artery pressure by 23 ± 6% ($P < 0.05$ compared to infusion baseline), but had no effect on mean arterial blood pressure or systemic vascular resistance (Figure 11). The decrease in pulmonary artery pressure with nitrite nebulization was associated with a progressive increase in exhaled NO from 4.8 ± 1.2 to 10.1 ± 2.0 ppb ($P < 0.05$ compared to baseline, Figure 11). Figure 2 shows a comparison of the effects of nitrite inhalation after 20 minutes on hypoxic versus drug-induced normoxic pulmonary vasoconstriction. The changes in mean pulmonary artery pressure and exhaled NO were significantly larger with nitrite treatment during hypoxic conditions. Overall the effects of nitrite inhalation on normoxic (thromboxane-induced) pulmonary hypertension were less than those observed with hypoxic pulmonary hypertension (Figures 10, 11, 12A), consistent with a model of hypoxemic and possibly acidemic potentiation of nitrite's vasoactivity.

pH and oxygen dependence of the nitrite reductase activity of deoxyhemoglobin

We hypothesize that the biochemical conversion of nitrite to NO requires both deoxyhemoglobin and protonation. Thus, data from both the normoxic and hypoxic experiments were used to study the influence of hemoglobin saturation and pH on NO production from nitrite. Measurements of exhaled NO gas and NO-modified hemoglobin (iron-nitrosyl-hemoglobin) were used as both dosimeters of NO production and as a measure of the direct byproducts of the nitrite reductase reaction of nitrite and hemoglobin to produce NO. Figure 12 shows that iron-nitrosyl-hemoglobin, measured by tri-iodide based reductive chemiluminescence (Figure 12B) and electron paramagnetic resonance (Figure 12C), was markedly increased by nitrite inhalation during hypoxia but not with drug-induced normoxic pulmonary vasoconstriction. As shown in Figure 12D, change in mean pulmonary artery pressure during hypoxia after inhalation of nebulized sodium nitrite was related to blood pH, with increased vasodilation associated with decreasing pH ($r = 0.57$ $P = 0.055$).

Comparison with inhaled NO and duration of effect.

We next compared the efficacy of nitrite with the current therapeutic standard, inhaled NO gas. After initiation of hypoxia, lambs were subjected to (20 ppm) inhaled NO gas or nebulized nitrite for 20 minutes. The data in Figure 13 show the duration and magnitude of the effect of NO gas inhalation (Figure 13A) or nitrite nebulization (Figure 13B, 13C) on hemodynamic and metabolic measurements during hypoxia. Although both treatments resulted in a pronounced reduction in hypoxic pulmonary hypertension, the response to inhaled NO gas was slightly more rapid and pulmonary pressure more nearly approached baseline when contrasted to the 60-70% correction in pressure elicited by nitrite. Systemically, mean arterial blood pressure and resistance was reduced to a similar extent with both treatments during hypoxia. However, with the relative chemical stability of the nitrite anion compared with NO gas, there was sustained vasodilation for more than 60 minutes

(the duration of the hypoxic challenge) after discontinuation of nitrite inhalation, whereas the termination of NO gas delivery abolished the vasodilating effect in a matter of seconds (Figure 13A, 13B). The relatively sustained effect of nitrite nebulization might be therapeutically advantageous by allowing for intermittent therapy analogous to the treatment of asthma with beta-adrenergic agonists by meter dose inhaler. The time course of nitrite inhalation-induced pulmonary vasodilation and plasma nitrite levels are shown (Figure 13C, 13D). In this experiment which tracked biochemical changes for a longer period that in Figure 10 methemoglobin (MetHb) concentrations increased from 2.1 ± 0.1 % during baseline to 2.8 ± 0.2% after nitrite nebulization ($P < 0.05$).

Discussion

A principle finding of this example is that a brief period of inhalation of nebulized sodium nitrite solution produces rapid and selective pulmonary vasodilation during hypoxic-induced pulmonary hypertension in newborn lambs. The significant reduction in pulmonary artery pressure following nitrite nebulization was sustained when hypoxia was continued for more than an hour after termination of nitrite nebulization. In none of the experiments did nitrite inhalation produce systemic hypotension, and methemoglobin elevation was minimal. From a mechanistic standpoint, nitrite administration was associated with NO production, measured by exhaled NO gas and NO-modified hemoglobin, with responses in proportion to levels of hemoglobin-oxygen desaturation and decreases in blood pH. These data support the paradigm that nitrite is an NO-dependent vasodilator whose bioactivation is coupled to hemoglobin deoxygenation and protonation.

Inhaled NO gas is the current standard for the treatment of pulmonary hypertension. Figure 13 provides a comparison of the effects of NO gas at 20 ppm with those of aerosolized nitrite. In about 5 minutes the NO gas effectively ablated about 80% of hypoxic-induced pulmonary hypertension, an effect that was short lived but which could be reproduced when it was given again 20 minutes later. Aerosolized sodium nitrite removed about 60% of hypoxic-induced pulmonary hypertension. This response was consistently observed in each of the lambs studied and it persisted throughout the one-hour period of hypoxia that was maintained after the nitrite aerosol was discontinued. The changes in pulmonary blood flow were accompanied by corresponding changes in the calculated resistance to blood flow through the lungs, indicating that changes were in the pulmonary vasculature rather than secondary to changes in cardiac output or systemic effects that might have altered perfusion pressures.

We demonstrate herein that aerosolized nitrite is an NO producing agent in the newborn lamb that can be readily administered by nebulization and appears to exhibit a wide therapeutic-to-safety margin, with limited systemic hemodynamic changes and methemoglobin production. This presents an attractive therapeutic option to inhaled NO. Nitrite is an ideal "NO producing" agent in that it 1) is a naturally occurring compound in blood, alveolar lining fluid, and tissue, and 2) has no parent-compound leaving group, such as the diazenium diolates, that requires extensive toxicological study prior to translation to human disease, and 3) it is already approved for human use in cyanide antidote kits. These advantages are to be counterbalanced against possible problems that might occur

with more prolonged delivery, including alveolar nitrite accumulation, systemic vasodilation, and the development of methemoglobinemia.

In conclusion, the data presented in this example suggest that inhaled nitrite is a potent and selective vasodilator of pulmonary circulation of the newborn lamb and further support the paradigm that nitrite, and particularly salts of nitrite, such as sodium nitrite, is an NO-dependent vasodilator whose bioactivation is coupled to hemoglobin deoxygenation and protonation. In none of our studies did inhaling nitrite produce systemic hypotension or elevate methemoglobin levels.

Example 4

Use of nitrite infusions for the prevention of cerebral artery vasospasm after subarachnoid hemorrhage

This example describes a method for using nitrite infusion to prevent cerebral artery vasospasm after intracranial hemorrhage.

Subarachnoid hemorrhage (SAH) due to the rupture of intracranial aneurysms affects 28,000 Americans annually. Almost 70% of patients with aneurysmal SAH develop severe spasm of the cerebral arteries on the seventh day after SAH. Despite aggressive medical therapy, neurological deficits resulting from vasospasm continue to be a major cause of morbidity and mortality. Although the etiology of cerebral vasospasm is poorly understood, there is increasing evidence that erythrocyte hemolysis in the cerebrospinal fluid and decreased availability of nitric oxide (NO), a potent vasodilator, plays a significant role. Reversal of vasospasm by NO or NO prodrugs has been documented in several animal models.

Despite half a century of research and clinical trials, delayed cerebral vasospasm (DCV) remains the single cause of permanent neurological deficits or death in at least fifteen percent of patients following otherwise successful endovascular or surgical treatment for ruptured intracranial aneurysm. Decreased bioavailability of nitric oxide (NO) has been mechanistically associated with the development of DCV. This work was carried out to determine whether infusions of nitrite, a naturally occurring anion that reacts with deoxyhemoglobin to form NO and S-nitrosothiol, might prevent DCV via reactions with perivascular hemoglobin.

Methods

An autologous arterial blood clot was placed around the right middle cerebral artery (R MCA) of 14 anesthetized *Cynomolgus* monkeys at day 0. Sodium nitrite solution ($NaNO_2$, 135 mg/daily and 180 mg/daily, which approximates 45 mg/kg and 60 mg/kg per day) in 0.9% saline (n=6) or saline alone (n=8) was infused intravenously for 14 days in awake animals via an ambulatory MiniMed Infusion Pump, at 2μl/minute. Cerebral arteriogram was performed before clot placement and on days 7 and 14, for assessment of DCV. Arteriographic vasospasm was defined as a 25% or greater reduction in the proximal 14 mm of the R MCA area as measured on the AP projection of the cerebral arteriogram (blinded assessment). Mean arterial blood pressure was

measured and blood samples were collected daily from day 0; the cerebral spinal fluid samples were collected on day 0, 7, and 14.

Results

In control animals, cerebral spinal fluid nitrite levels decreased from 3.1±1.5 μM to 0.4±0.1 μM at 7 days and 0.4±0.4 μM at 14 days (Figure 14), and all eight animals developed significant vasospasm of the R MCA (Figures 15 and 16), complicated by stroke and death in one animal.

Nitrite infusions were associated with increases in plasma cerebrospinal fluid nitrite and blood methemoglobin concentrations without systemic hypotension (Figure 14), and significantly reduced the severity of vasospasm (Figures 15 and 16; no animals developed significant vasospasm; mean reduction in the R MCA area on day 7 after SAH was 8±9% versus 45±5%; $P < 0.001$). Pharmacological effects of nitrite infusion were associated with bioconversion of cerebrospinal fluid nitrite to S-nitrosothiol, a potent vasodilating NO donor intermediate of nitrite bioactivation. There was no clinical or pathological evidence of nitrite toxicity.

Conclusions

Subacute sodium nitrite infusions prevent DCV in a primate model of SAH, and do so without toxicity. These data evince a novel, safe, inexpensive, and rationally designed therapy for DCV, a disease for which no current preventative therapy exists.

While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments, and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

CLAIMS

1. A method for treating or ameliorating a condition selected from:

(a) hepatic or cardiac or brain ischemia-reperfusion injury;

(b) pulmonary hypertension; or

(c) cerebral artery vasospasm,

in a subject by decreasing blood pressure and/or increasing vasodilation in the subject, the method comprising administering sodium nitrite to the subject to decrease the blood pressure and/or increase vasodilation in the subject, thereby treating or ameliorating the condition.

2. The method of claim 1, which is a method for treating or ameliorating hepatic or cardiac or brain ischemia-reperfusion injury.

3. The method of claim 2, wherein administering sodium nitrite to the subject is intravenous.

4. The method of claim 2 or 3, wherein the sodium nitrite is administered to a circulating concentration of about 0.6 to 240 μM.

5. The method of claim 1, which is a method for treating or ameliorating pulmonary hypertension.

6. The method of claim 5, wherein the pulmonary hypertension is neonatal pulmonary hypertension.

7. The method of claim 5 or 6, wherein administering sodium nitrite to the subject is by inhalation.

8. The method of claim 7, wherein the sodium nitrite is nebulized.

9. The method of any one of claims 5 through 8, wherein the sodium nitrite is administered at a rate of 270 μmol/minute.

10. The method of claim 1, which is a method for treating or ameliorating cerebral artery vasospasm.

11. The method of claim 10, wherein administering sodium nitrite to the subject is intravenous.

12. The method of claim 10 or 11, wherein the sodium nitrite is administered at a rate of about 45 to 60 mg/kg.

13. The method of any one of claims 1-12, wherein the sodium nitrite is administered in combination with at least one additional agent.

14. The method of any one of claims 1-13, wherein the subject is a mammal.

15. The method of any one of claims 14, wherein the subject is a human.

Figure 1A

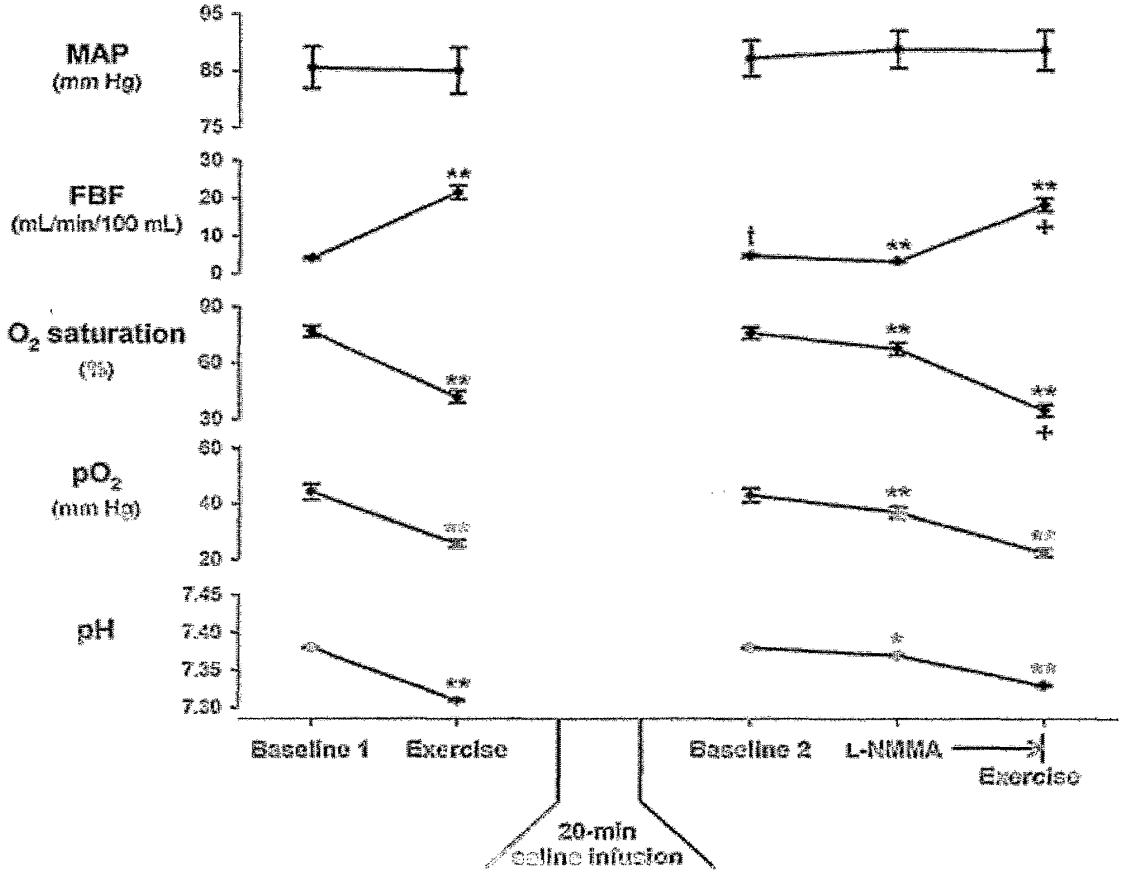


Figure 1B

Figure 2A

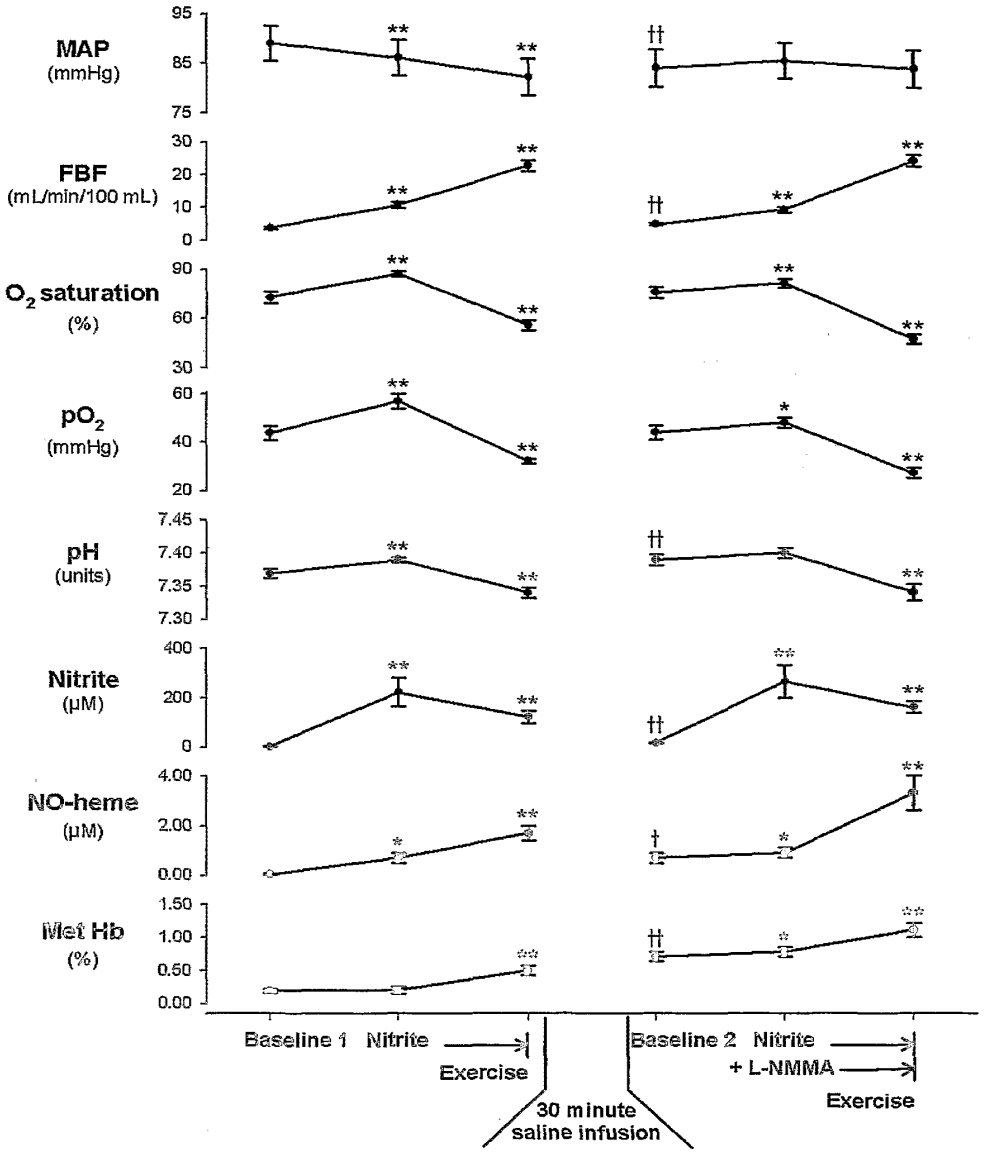


Figure 2B

Figure 3A

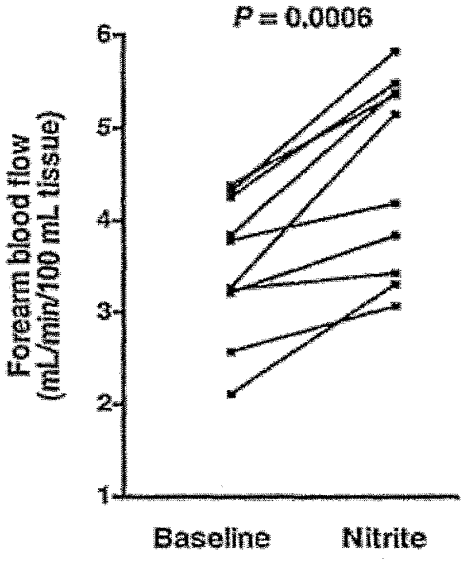


Figure 3B

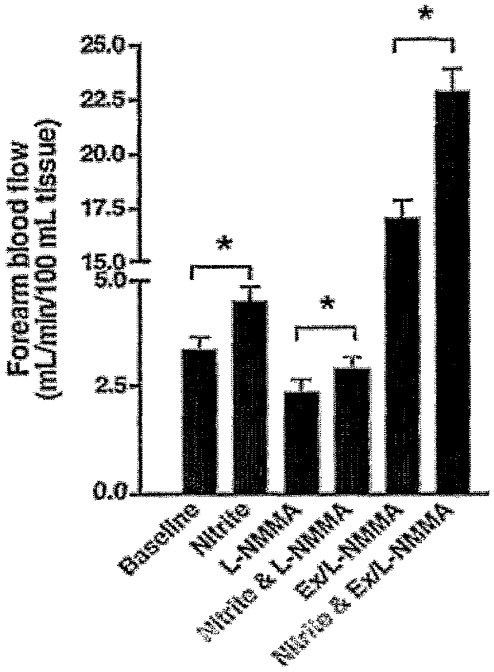


Figure 3C

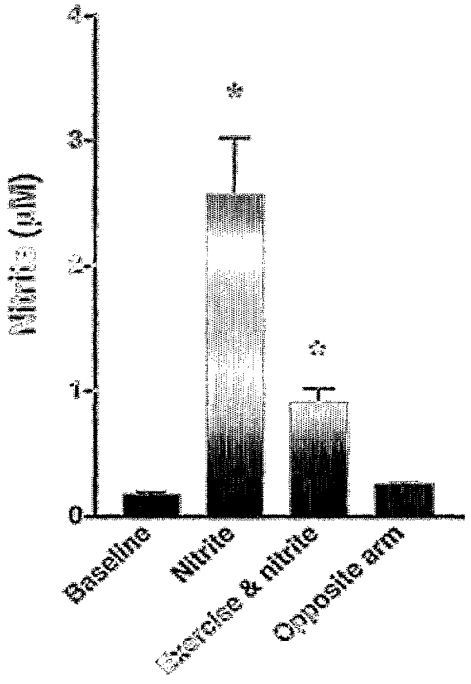


Figure 3D

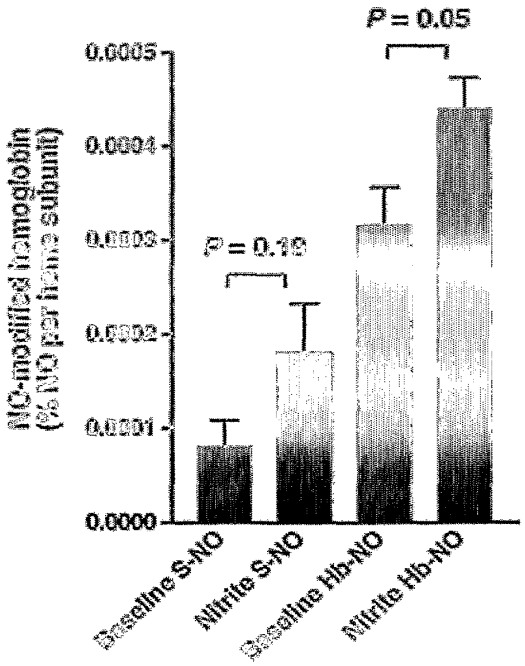


Figure 4A

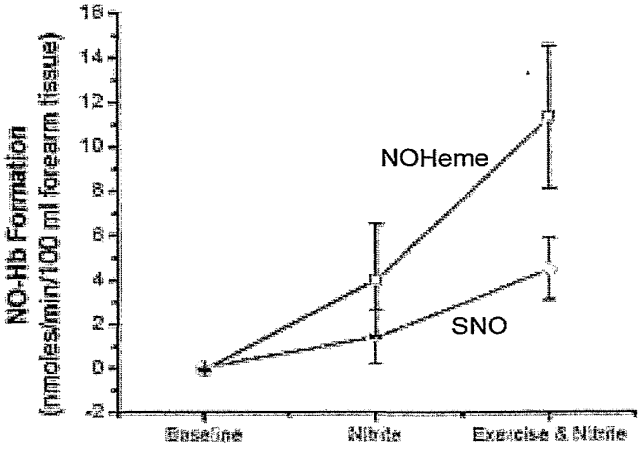


Figure 4B

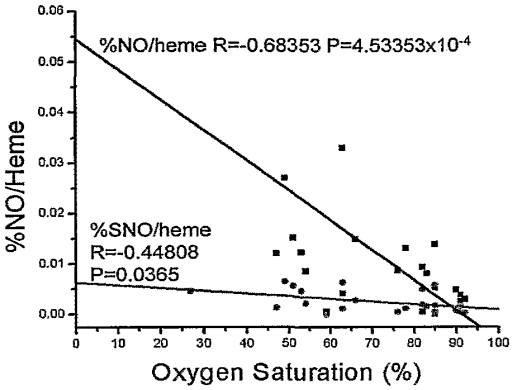


Figure 5A

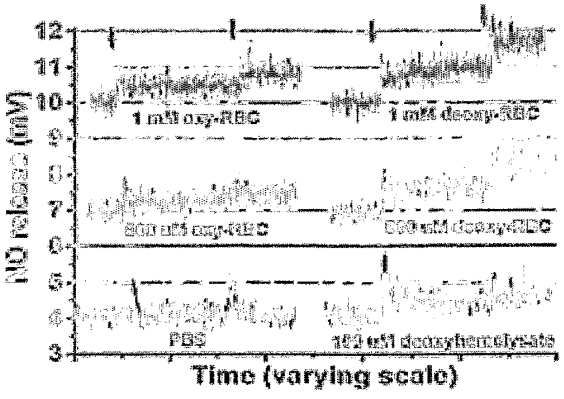


Figure 5B

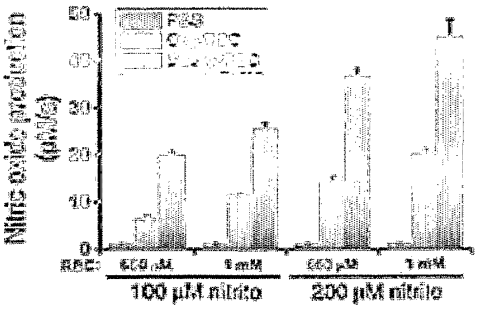


Figure 6A

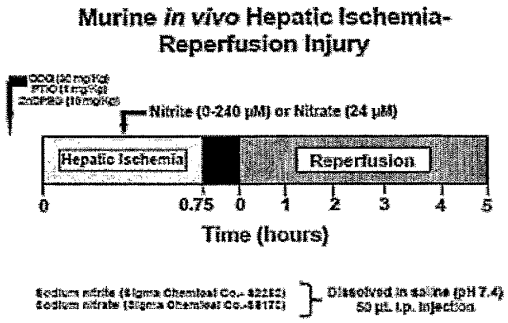


Figure 6B

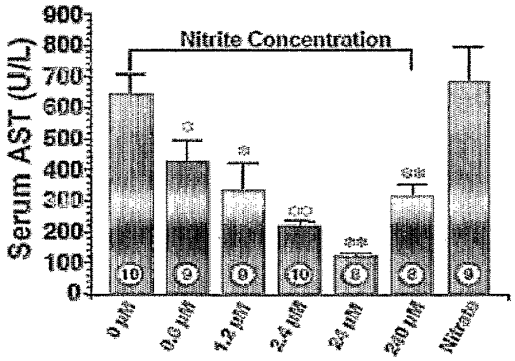


Figure 6C

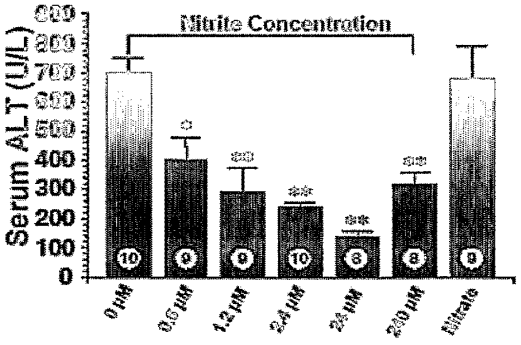


Figure 6D

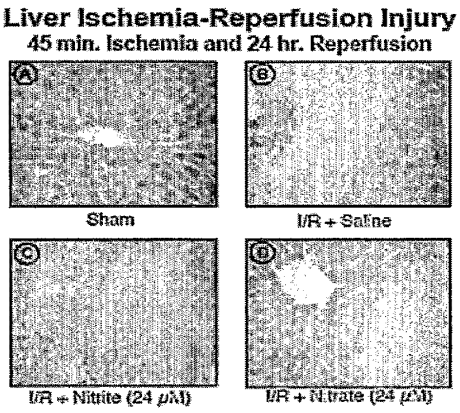


Figure 6E

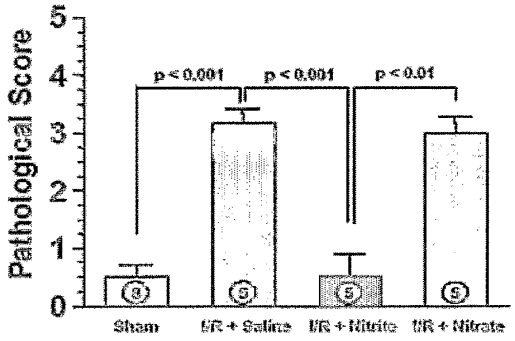


Figure 6F

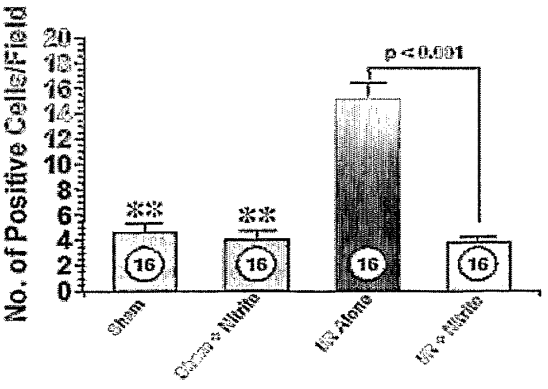


Figure 7A

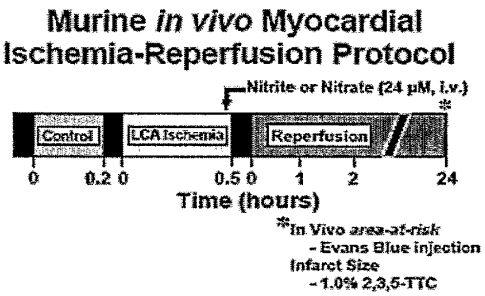


Figure 7B

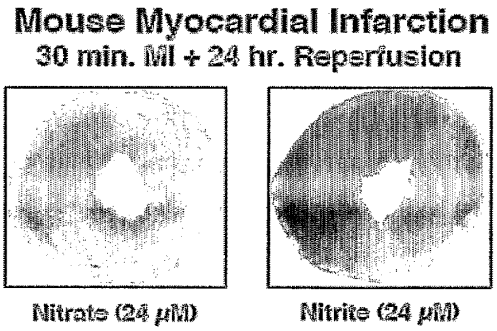


Figure 7C

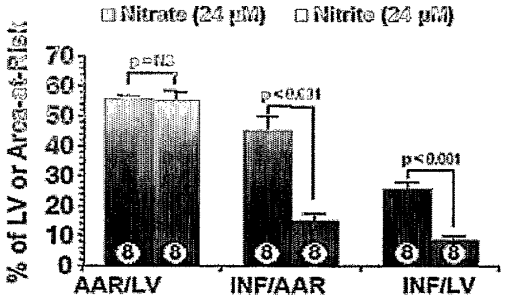


Figure 7D

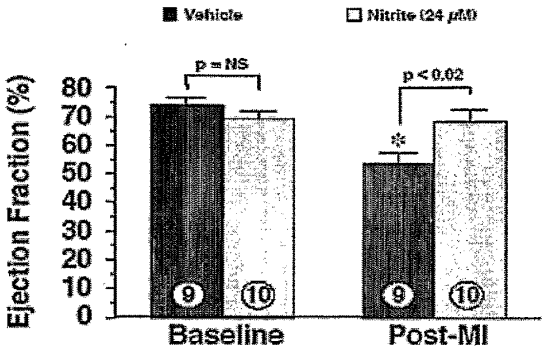


Figure 7E

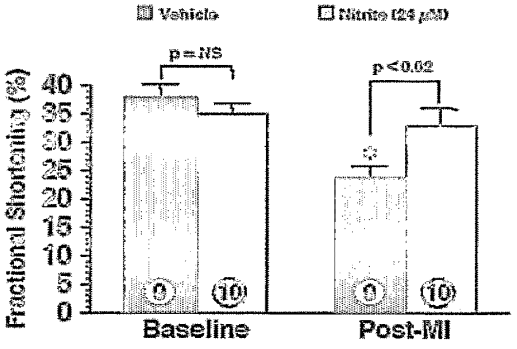


Figure 8A

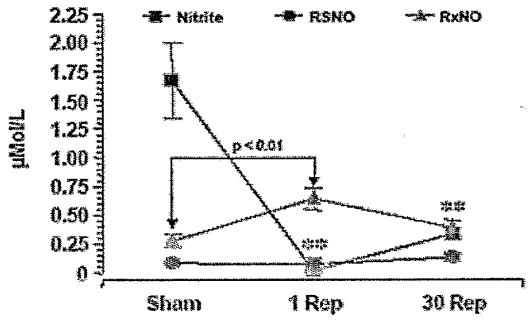
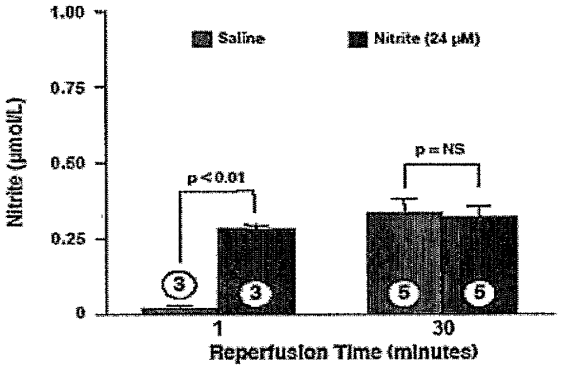


Figure 8B



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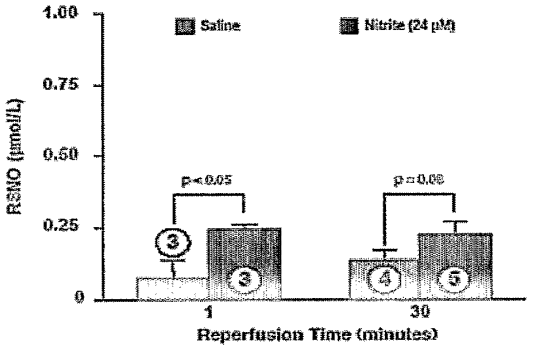

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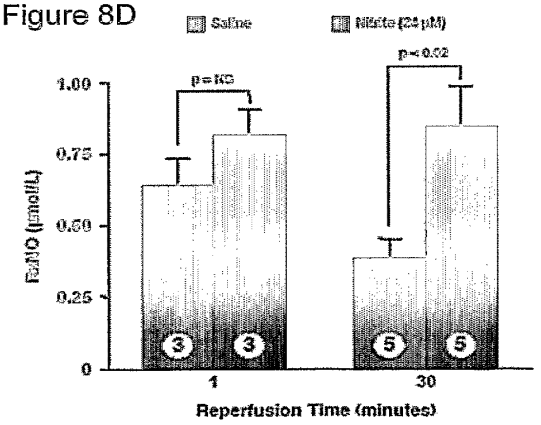


Figure 9A

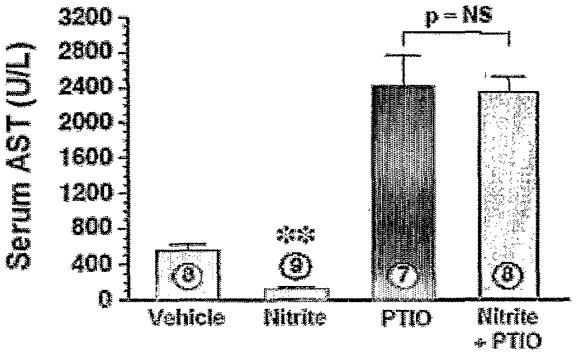


Figure 9B

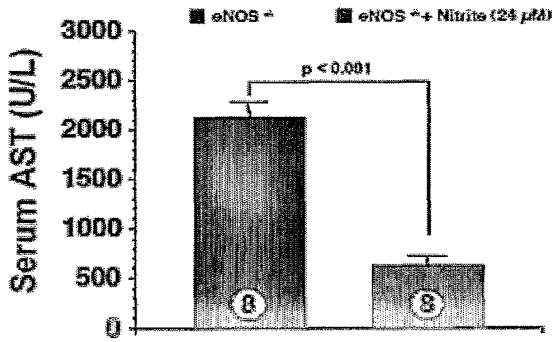


Figure 9C

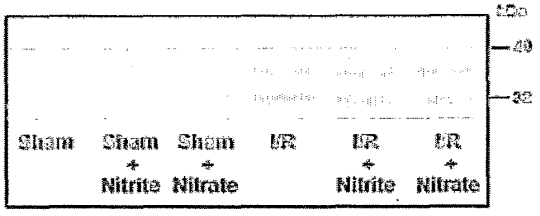


Figure 9D

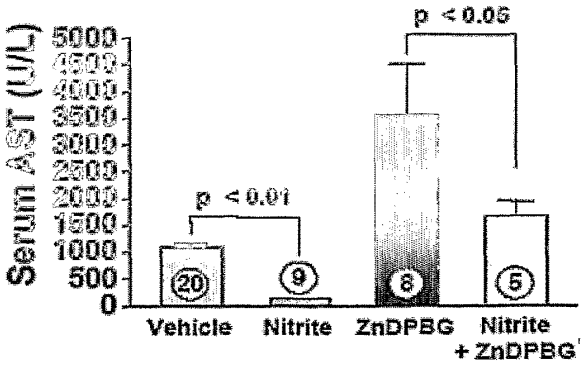


Figure 10A

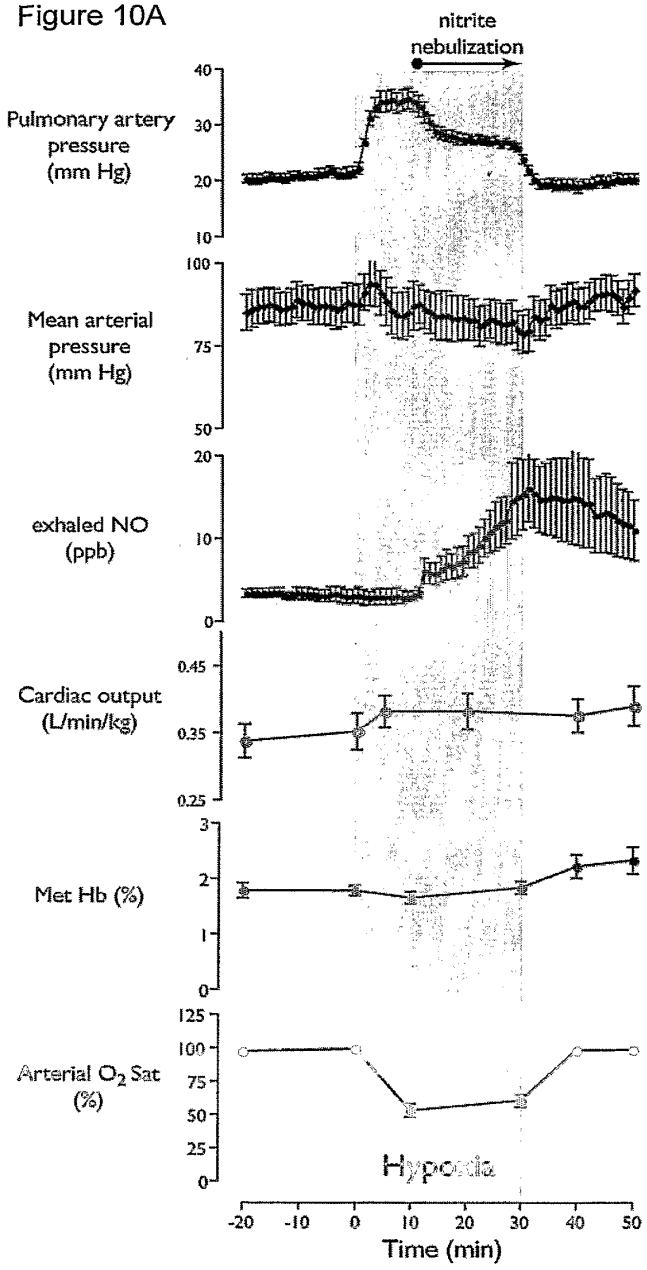
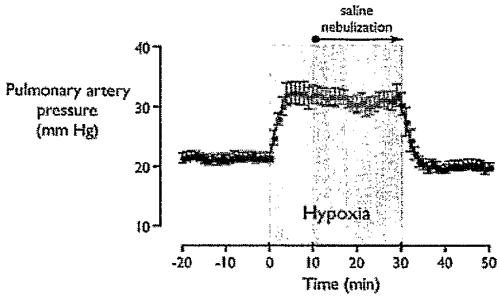


Figure 10B



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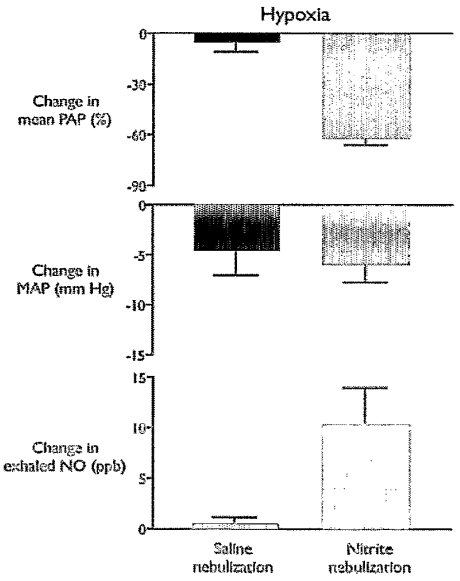


Figure 11

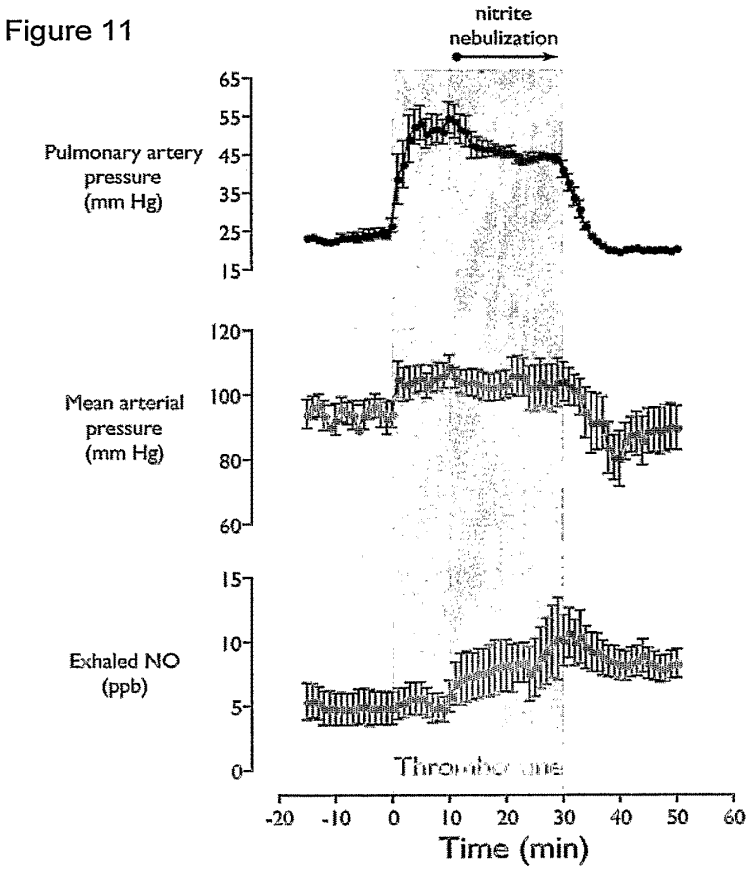

nitrite
nebulization
Pulmonary artery
pressure
(mm Hg)
65
55
45
35
25
15
Mean arterial
pressure
(mm Hg)
120
100
80
60
Exhaled NO
(ppb)
15
10
5
0
-20
-10
0
10
20
30
40
50
60
Time (min)

Figure 12A

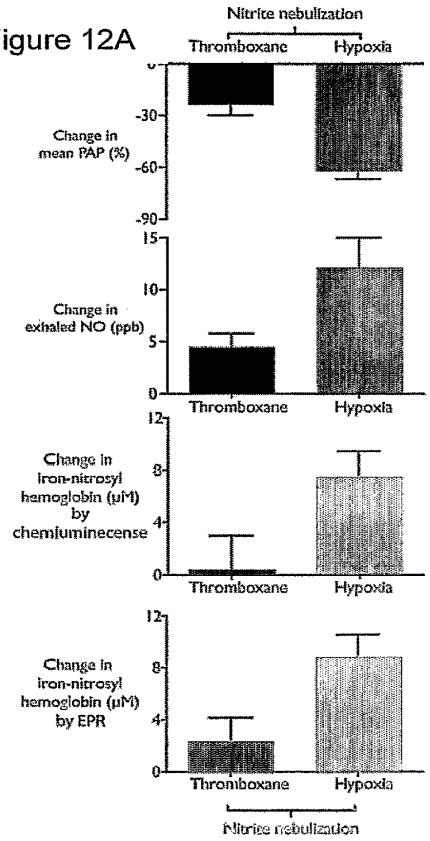


Figure 12B

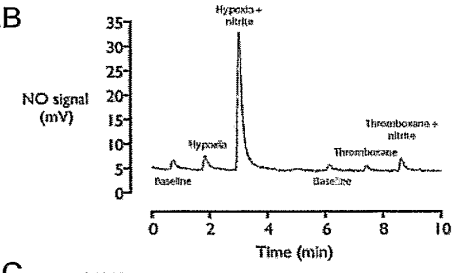


Figure 12C

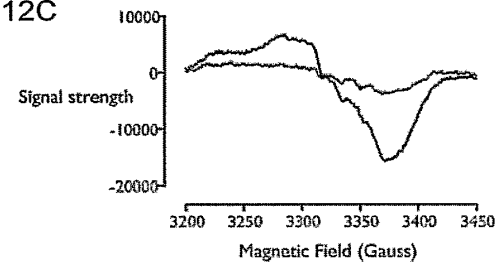


Figure 12D

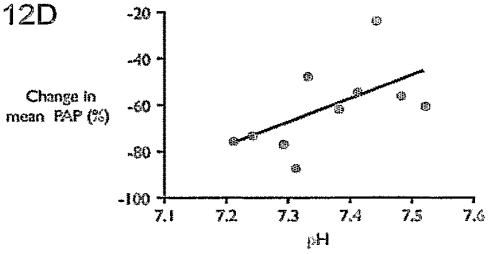


Figure 13A

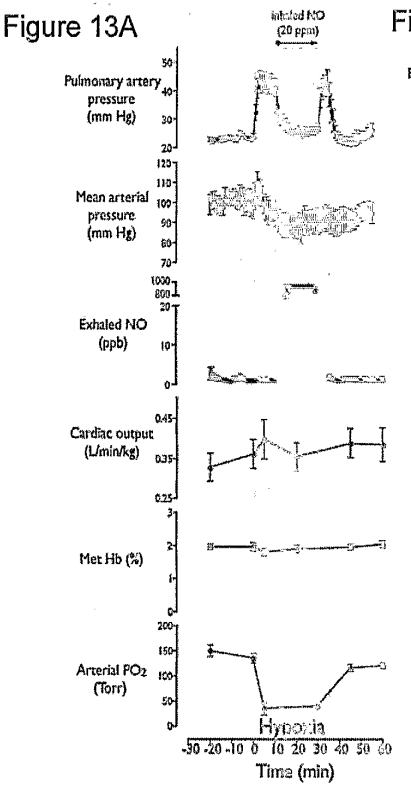


Figure 13B

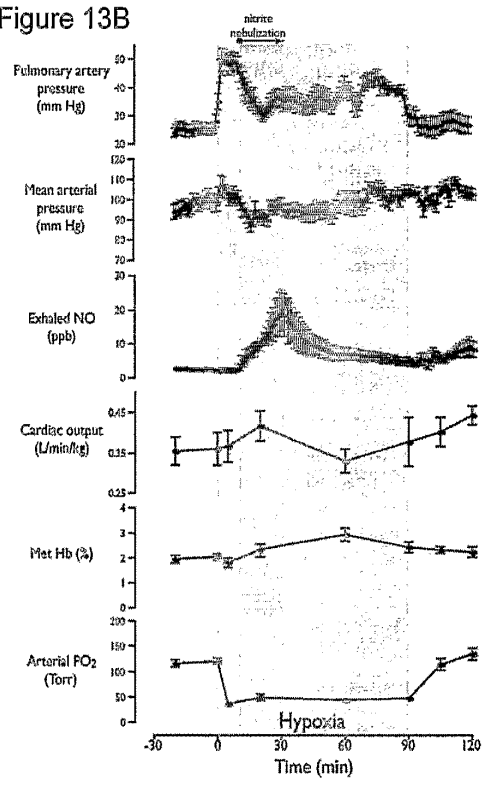


Figure 13C

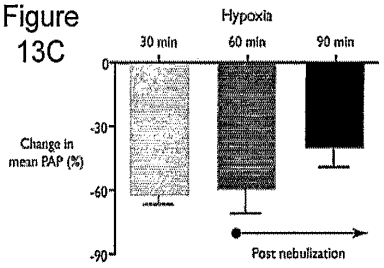


Figure 13D

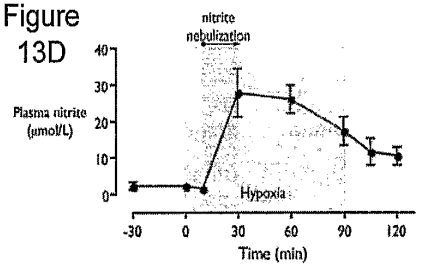


Figure 13E

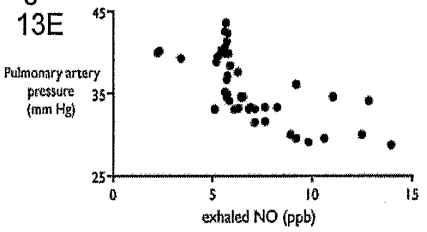
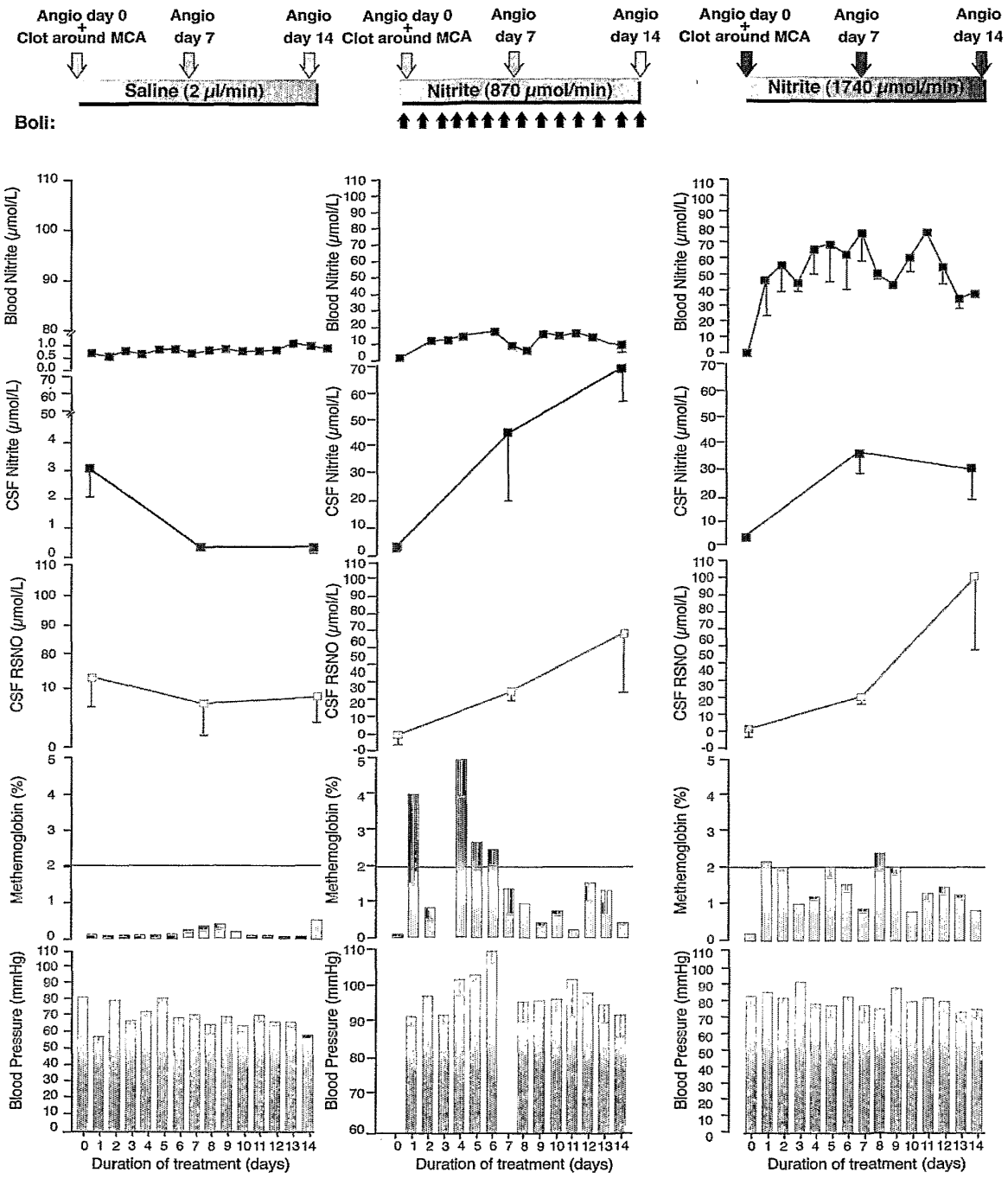


Figure 14


Angio day 0
+
Clot around MCA
Angio
day 7
Angio
day 14
Saline (2 µl/min)
Nitrite (870 µmol/min)
Nitrite (1740 µmol/min)
Boli:
Blood Nitrite (µmol/L)
CSF Nitrite (µmol/L)
CSF RSNO (µmol/L)
Methemoglobin (%)
Blood Pressure (mmHg)
Duration of treatment (days)

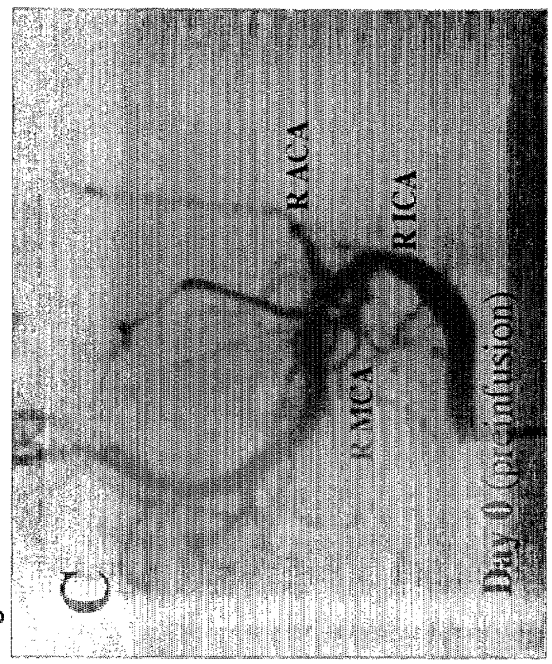


Figure 15C

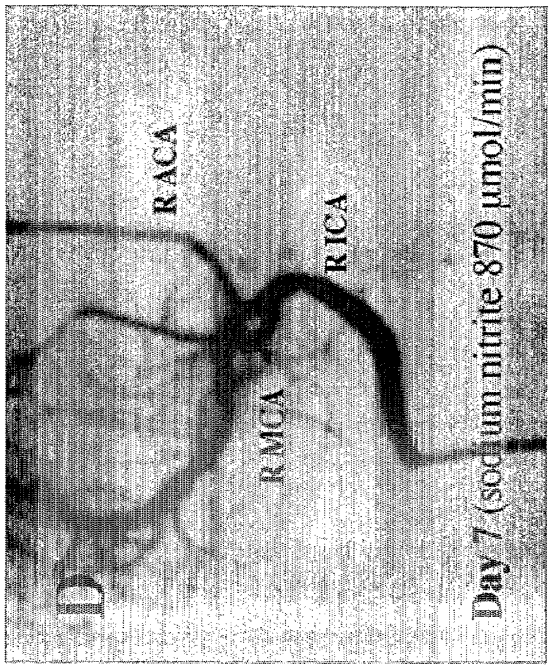


Figure 15D

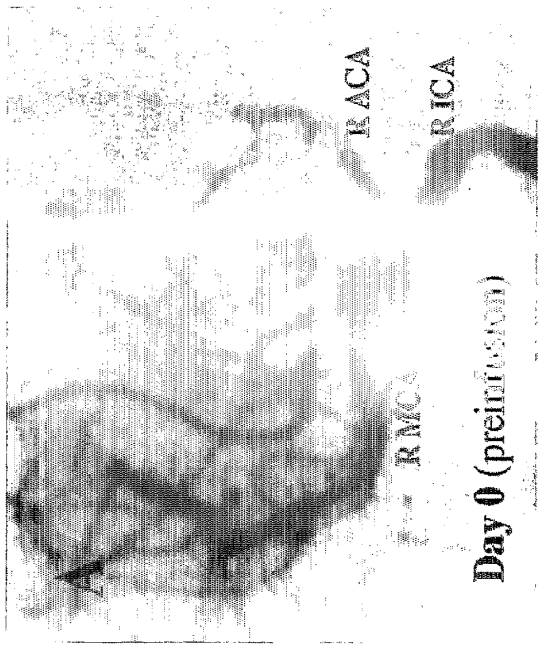


Figure 15A

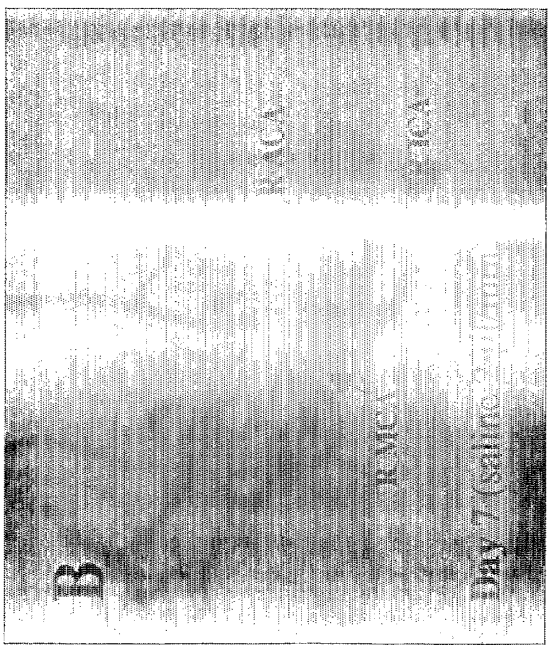


Figure 15B

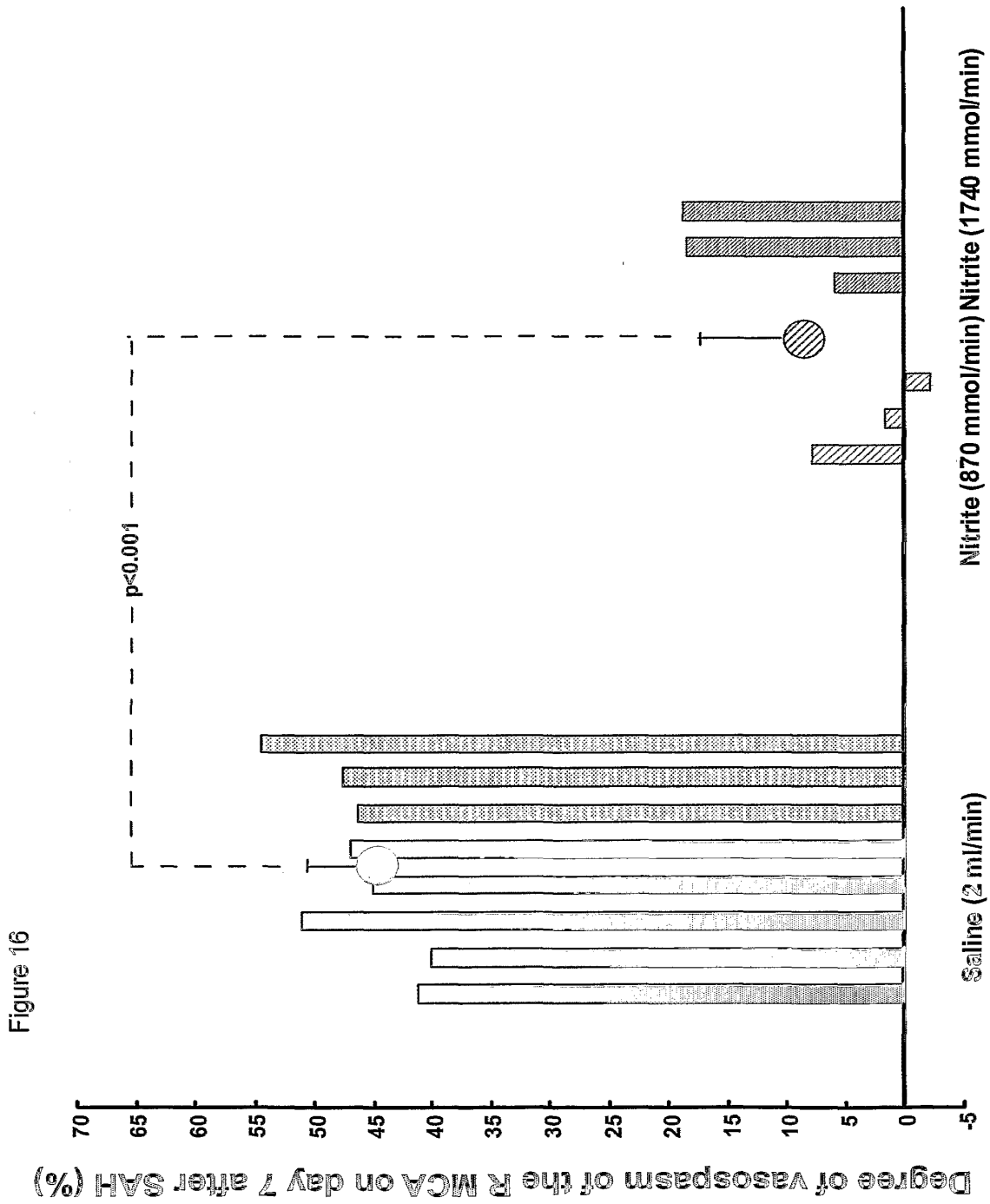

Degree of vasospasm of the R MCA on day 7 after SAH (%)
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
-5
p<0.001
Saline (2 ml/min)
Nitrite (870 mmol/min)
Nitrite (1740 mmol/min)

Figure 16

WO2006127907

Publication Title:

LOCALIZED DELIVERY OF CARDIAC INOTROPIC AGENTS

Abstract:

Abstract of WO 2006127907

(A2) Translate this text The present invention provides novel methods for the localized delivery of inotropic agents to the heart, including specific regions of the heart, such as the ventricles, for example in a subject undergoing cardiothoracic surgery, with the aim of supporting the myocardial contractile function of the heart.

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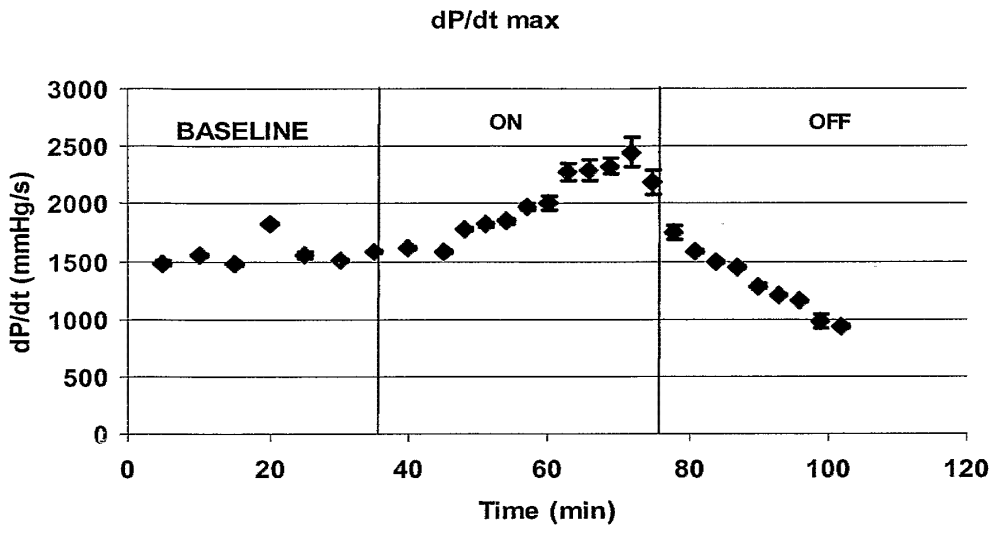
(54) Title: LOCALIZED DELIVERY OF CARDIAC INOTROPIC AGENTS



(57) Abstract: The present invention provides novel methods for the localized delivery of inotropic agents to the heart, including specific regions of the heart, such as the ventricles, for example in a subject undergoing cardiothoracic surgery, with the aim of supporting the myocardial contractile function of the heart.

WO 2006/127907 A2

LOCALIZED DELIVERY OF CARDIAC INOTROPIC AGENTS

CROSS REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application Serial No. 60/684,594 filed May 25, 2005, the contents of which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

[002] The present invention is directed to methods for the localized delivery of inotropic agents to the heart, including specific regions of the heart such as the ventricles, in a subject in need of such contractile support.

BACKGROUND OF THE INVENTION

[003] Performance of cardiac surgery is a delicate and invasive procedure. The majority of epicardial bypass graft surgeries, and all open heart procedures, require temporary arrest of the heart to allow the surgeon to accomplish the required task without interference from heart movement. An extracorporeal machine, known as a cardiopulmonary bypass (CPB) circuit, assumes the heart and lungs' role of supplying oxygenated blood to the rest of the body while the heart is arrested. Once the surgery is completed, the heart must be re-started, and the patient weaned from the CPB.

[004] While the use of CPB makes cardiac surgery feasible, it is also associated with significant risks and difficulties. The use of a CPB machine usually requires an aortic cross-clamp to separate the heart from the rest of the circulation. Because the coronary arteries arise very close to the heart, the cross clamp must be applied distal to their ostia and therefore they receive no blood flow for prolonged periods, and thus the heart becomes ischemic. Despite numerous myocardial protection strategies, such as hypothermia and chemical cardioplegia to decrease oxygen consumption by arresting the heart, many patients' heart function is

significantly impaired by both chemical arrest and the CPB circuit itself. Chemical cadioplegia, altered coronary perfusion, embolic events and direct manual manipulation of the heart during the procedure all contribute to depression of myocardial function after it is restarted. Furthermore, the degree of post CPB dysfunction may depend on the duration of the CPB time. Patients emerge from chemical cardiac arrest with a spectrum of left ventricular dysfunction, from transient mild impairment to outright ventricular failure and inability to be separated from the CPB. Patients with preexisting ventricular dysfunction are at the greatest risk for further myocardial impairment during CPB.

[005] Moreover, because of improvements in surgical technique and intraoperative myocardial protection, as well as the increasing availability of sophisticated valvular, direct myocardial resections, repairs of septal defects, and coronary bypass procedures, more cardiac operations are being performed on patients with more advanced stages of disease and decreased ventricular function. Indeed, the number of operative risk factors, including advanced age, female gender, severity of angina, triple vessel disease, and left ventricular dysfunction, has increased among patients currently undergoing coronary artery bypass surgery [Davis PK, et al., Ann Thorac Surg 1989; 47:493-98].

[006] In addition, there are important, potentially damaging effects of CPB itself on the cardiovascular system, including increased capillary permeability with attendant transcapillary plasma loss, renal dysfunction, peripheral or central vasoconstriction, coagulopathy, platelet destruction and dysfunction, and destruction of red blood cells [Kalter RD, et al., .J Thorac Cardiovasc Surg 1979; 77:428-35; Kirklin JK, et al., J. Thorac Cardiovasc Surg 1983; 86:845-57.]. Patients with preexisting cardiomyopathies are at even greater risk for postoperative contractile dysfunction. These effects are often transient, but their timing and intensity can make it difficult to impossible in many instances to separate the patient from the CPB circuit.

[007] Weaning a patient off cardiopulmonary bypass (CPB) is a critical step of cardiac surgical procedures. Restarting the heart and returning it and the lungs to the circulation after CPB carries the potential to severely stress an already compromised heart. In the best of circumstances, weaning off CPB can be a relatively straightforward process that requires reestablishing ventilation to the lungs and slowly lowering the circulatory support from the CPB pump. In a significant number of cases however, weaning is especially difficult, and in a few situations simply impossible.

[008] Current available options to support patients who fail to wean from CPB, in order of increasing invasiveness and associated morbidity, include intravenous infusion of inotropes that enhance myocardial contractility, insertion of an intra-aortic balloon pump to augment coronary perfusion and diminish the workload on the heart, and placement of a ventricular assist device. However, each of these treatments is accompanied by significant morbidity and technical limitations, and potential toxicity. Examples of limitations associated with such treatments include proarrhythmic and systemic effects from systemic infusion of inotropes, damage from large-bore indwelling vascular access, need for patient immobility and sedation, as well as risks associated with the placement of a large mass of foreign materials with externalized connections. The pumps and devices have high rates of infection and thromboembolic complications, and require patient immobility, sedation, sometimes prolonged postoperative ventilation, and the most extreme of intensive care nursing support. Weaning of small children after prolonged, difficult and complex operations can represent a further challenge to the surgical team as assist devices may not be readily available in appropriate sizes.

[009] One of the significant challenges of supporting patients as they transition from CPB to the intensive care unit is the variability between patients regarding the timing and degree of support each patient requires. Many patients only need short-term inotropic support to help them transition from CPB to the intensive care unit, while the support required by other patients is much more extensive and

potentially associated with greater risks. Thus, it would be desirable to have less intrusive means that could be used to support these patients as they transition off CPB.

[0010] Inotropic agents are one approach used to enhance a high-risk patient's ability to wean from CPB. Pharmacologic inotropic agents enhance myocardial contractility, and fall into two broad categories: sympathomimetics such as epinephrine (adrenaline), norepinephrine (noradrenaline), dobutamine, isopreterenol, salbutamol, salmeterol, terbutaline, isoproterenol, phenylephrine, ephedrine, clonidine and dopamine, and phosphodiesterase inhibitors such as milrinone and amrinone. Each of these compounds, while increasing the inotropic state of the heart, has limitations that restrict the doses that can be given intravenously and often necessitate infusion of additional agents to counteract side effects. For example, dopamine dosing is limited by the increase in the rate and irritability of electrical excitation of the heart that accompanies the desired inotropic effect. Alternatively, phosphodiesterase inhibitors increase intracellular cyclic AMP, an intracellular signaling molecule that increases inotropy, but unfortunately dilates arterioles and causes systemic vasodilation and hypotension. As a result, vasoconstricting sympathomimetic agents often need to be co-administered and these again can lead to proarrythmogenic states and undesirable tachycardia.

[0011] One important consideration of the use of inotropic agents is that they are administered systemically and thus treat all vascular beds. Systemic side effects of sympathomimetics include potential renal and cerebral vasoconstriction, and pulmonary artery hypertension, which in turn can induce right heart failure. Other undesired effects are excess tachycardia and electrical irritability.

[0012] Accordingly, there is a need for improved methods to support patients as they transition off CPB, by improving contractile function of the heart without extraventricular effects, such as tachycardia, vasoconstriction or systemic hypotension.

SUMMARY OF THE INVENTION

[0013] The present invention provides novel methods for the localized delivery of inotropic agents to the heart, including specific regions of the heart, such as the ventricles, in a subject in need thereof.

[0014] Support of the weakened heart such as occurs while a patient is coupled to a CPB circuit, and while the patient transitions off CPB, is critical to recovery from cardiac surgery. We have discovered methods to take advantage of existing polymeric controlled release strategies to deliver inotropic agents directly or indirectly to the heart, preferably directly, including to specific regions of the heart. By locally delivering the inotropic agent directly to the heart, the systemic exposure of the inotropic agents is limited, avoiding the alterations in vascular tone, and heart rate and electrical excitability associated with systemic administration of these agents.

[0015] The methods of the present invention can be used to treat any patient in need of transient contractile support to the heart, where such support can be provided by the local delivery of inotropic agents either directly or indirectly to the heart, including specific regions of the heart, such as the ventricles. One would apply the agent through the cardiac blood stream, or preferably directly in the heart. The agent can be applied through the coronary artery or vein and onto the heart surface. The agent can also be applied through the ventricular or atrial walls and onto the heart surface. The agent can also be applied through direct and extensive surgical field exposure, minimally invasive exposure via a pericardial window or heart port, or percutaneous or endovascular catheters.

[0016] In one embodiment, the patient is in need of localized delivery of an inotropic agent to provide contractile support as a result of a surgical intervention. Surgical interventions include but are not limited to cardiac surgery, thoracic surgery,

and general surgery. In another embodiment, the patient is in need of transient localized delivery of an inotropic agent to provide contractile support as a result of trauma, shock, or heart failure.

[0017] In another embodiment, the patient is in need of transient inotropic support following an intervention less invasive than a major surgical intervention, referred to herein as a minimally invasive intervention. Such minimally invasive interventions include but are not limited to a percutaneous intervention or a catheter based intervention. In such embodiments, the inotropic agent can be delivered either from inside the heart chamber or from outside the heart.

[0018] One preferred embodiment provides transient localized delivery of inotropic agents to support the heart of a patient undergoing surgery. In one embodiment, the patient requires support from a cardiopulmonary bypass (CPB) circuit. In another embodiment, the patient does not require support from a CPB circuit. In one particularly preferred embodiment, the patient is a cardiac patient.

[0019] The present invention provides the local delivery of any inotropic agent, including but not limited to sympathomimetics and phosphodiesterase inhibitors. Preferred sympathomimetics include epinephrine, norepinephrine, isoproterenol, dobutamine and dopamine, and analogues and derivatives thereof. Preferred phosphdiesterase inhibitors include milrinone and amrinone, and analogues and derivatives thereof.

[0020] Any delivery vehicle which can be loaded with an inotropic agent and directly applied to the heart can be used in the present invention. Delivery vehicles include drug-impregnated, coated or relasing sheets, patches, matrix, hydrogel, foam, gel, cream, spray, microshpere, microcapsule, composite and ointment. Certain preferred delivery vehicles are polymeric controlled release vehicles.

[0021] The delivery vehicle is loaded with the inotropic agent and locally applied to the heart using any route for application which allows its local application

to the heart. In one embodiment, the delivery vehicle may be applied directly to the exposed heart during a surgical intervention, for example before the pericardium or sternum is closed. In another embodiment, the delivery vehicle may be applied through a less direct route, including but not limited to a percutaneous application or an endovascular injection.

[0022] Certain embodiments of the invention provide further localization of the delivery of the inotropic agent. In one embodiment, the delivery vehicle is placed away from the sinoatrial node or the right atrium. A preferred placement of the delivery vehicle is on the left ventricular free wall or apex of the ventricle.

[0023] One particularly preferred embodiment provides local delivery of dopamine to the ventricle without targeting the sinus node in the right atrium, limiting the excessive tachycardia observed in high dose intravenous infusion of this agent.

[0024] Another embodiment of the invention provides the use of a non-permeable barrier on the surfaces of the heart not treated with the delivery vehicle, to achieve additional localization. In another embodiment of the invention, non-permeable barriers can be used to direct drug toward the myocardium and prevent the loss of drug to ventricular blood flow or pericardial fluid.

[0025] Preferably, the delivery methods of the present invention are administered to the subject for a short time, i.e. just long enough to support the heart until it recovers from its weakened condition. Administration of the inotropic agent may last for a few hours to days, for example up to 14 days. The delivery methods of the present invention can be used to treat the heart prior to surgery, during surgery, after surgery, and any combination thereof.

DESCRIPTION OF THE FIGURES

[0026] Figure 1: Figure 1 shows contractility of the heart (max dp/dt (mmHg/s)) over time in rats administered dobutamine, a non selective beta agonist inotropic agent, which was delivered directly to the left ventricular wall. Contractility

was significantly increased shortly after dobutamine was applied to the surface of the heart.

[0027] Figure 2: Figure 2 shows left ventricular systolic blood pressure over time in rats administered Dobutamine, a nonselective beta agonist inotropic agent, which was delivered directly to the left ventricular wall. Local pericardial delivery of dobutamine increased systemic blood pressure. It is known that intravenous infusion of inotropic agents reduce systemic blood pressure.

[0028] Figure 3: Figure 3 shows heart rate over time in rats administered Dobutamine, a non selective beta agonist inotropic agent, which was delivered directly to the left ventricular wall.

DETAILED DESCRIPTION OF THE INVENTION

[0029] The present invention provides novel methods for the localized delivery of inotropic agents to the heart, including specific regions of the heart, in a subject in need of transient contractile support. One embodiment provides localized delivery of inotropic agents to support the heart during or following cardiac surgery, including as a subject transitions off of a cardiopulmonary bypass (CPB) circuit.

[0030] The present invention provides advantages over known methods to support the weakened heart, such as while a cardiac surgery patient is coupled to a CPB circuit, and as a patient transitions off CPB. To avoid the adverse side effects associated with systemic delivery of positive inotropic agents, we have discovered methods to take advantage of existing polymeric controlled release strategies to locally deliver inotropic agents directly to the heart. By locally delivering the inotropic agent directly to the heart, the systemic exposure of the inotropic agents is limited, avoiding the peripheral arterial dilation and systemic hypotension associated with systemic administration of some of these agents, and the tachycardia and vasoconstriction associated with others. In addition, because the methods of the

invention deliver the positive inotropic agent directly to localized heart surface, lower amounts, but potentially high local concentrations, can be delivered.

[0031] The inventors of the present invention have surprisingly shown that inotropic agents, when applied directly to the heart rather than systemically, increase contractility of the heart and minimize systemic side effects such as the reduction in systemic blood pressure that is seen when certain inotropic agents, such as dobutamine, isoproterenol, milrinone, or amrinone are administered systemically. Thus, the inventors have shown that local delivery of inotropic agents mimimizes systemic side effects while improving contractile function of the heart.

[0032] In one embodiment, a method of locally delivering a cardiac inotropic agent to the heart of a subject is encompassed. This method comprises locally administering to a subject in need thereof a therapeutically effective amount of at least one inotropic agent.

[0033] In one embodiment, the inotropic compound is an agent that interacts with the sympathetic nervous system and modulates calcium entry, G-proteins, ATP, or GTP, wherein the inotropic agent is selected from the group consisting of sympathomimetic compounds, phosphodiesterase inhibitors, BNP, ANP, and digitalis glycosides, and derivatives and analogues thereof.

[0034] The inotropic agent may be a sympathomimetic compound selected from the group consisting of epinephrine, norepinephrine, dobutamine, isoproterenol, salbutamol, salmeterol, terbutaline, phenylephrine, ephedrine, clonidine and dopamine, and derivatives and analogues thereof.

[0035] Alternatively, the inotropic agent may be a phosphodiesterase inhibitor selected from the group consisting of milrinone and amrinone, and derivatives and analogues thereof.

[0036] The subject to be treated may be a surgical patient. Non-limiting examples of surgical patients are a cardiac surgery patient, a thoracic surgery patient, and a general surgery patient.

[0037] In one embodiment, the cardiac surgery patient is selected from the group consisting of a cardiac surgery patient requiring support from a cardiopulmonary bypass circuit and a cardiac patient not requiring support from a cardiopulmonary bypass circuit.

[0038] In another embodiment, the subject has a condition selected from the group consisting of trauma, shock, and congestive heart failure.

[0039] In one embodiment, the inotropic agent is locally delivered to the heart by administering the inotropic agent directly to the heart via an open surgical wound. Alternatively, the local delivery comprises administering said inotropic agent directly to the heart percutaneously.

[0040] A method of reducing postoperative complications of cardiopulmonary bypass (CPB) surgery in a subject is also encompassed in the present invention. This method comprises locally administering to a subject in need thereof an effective amount of an inotropic agent in conjunction with CPB surgery of said subject. The inotropic agent may be a sympathomimetic compound or a phosphodiesterase inhibitor.

[0041] The inotropic agent may be administered to said subject during a time period consisting of 1) prior to said CPB surgery; 2) during said CPB surgery; 3) subsequent to said CPB surgery; and 4) combinations thereof.

[0042] As used herein, a "therapeutically effective amount" of the inotropic agent is an amount that is sufficient to effect myocardial contractility.

[0043] The inotropic agent may be delivered locally to the heart by its inclusion in a delivery vehicle.

Subjects for Administration

[0044] The methods of the present invention can be used to treat any patient in need of transient contractile support to the heart, where such support can be provided by the local delivery of inotropic agents directly to the heart, including specific regions of the heart, such as the ventricles.

[0045] In one embodiment, the patient is in need of localized delivery of an inotropic agent to provide transient contractile support as a result of a surgical intervention. Surgical interventions include major surgeries, including but not limited to cardiac surgery, thoracic surgery, and general surgery. In another embodiment, the patient is in need of localized delivery of an inotropic agent to provide contractile support as a result of trauma, shock, or heart failure.

[0046] In another embodiment, the patient is in need of inotropic support following an intervention less invasive than a major surgical intervention, referred to herein as a minimally invasive intervention. Such minimally invasive interventions include but are not limited to a percutaneous intervention or a catheter based intervention. In such embodiments, the inotropic agent can be delivered either from inside the heart chamber or from outside the heart, as described in detail below.

[0047] One preferred embodiment provides localized delivery of inotropic agents to support a patient undergoing surgery. In one embodiment, the surgical procedure requires the use of a cardiopulmonary bypass (CPB) circuit. In another embodiment, the procedure does not require the use of a CPB circuit. In one particularly preferred embodiment, the patient is a cardiac patient.

[0048] In order to perform many surgical procedures it is necessary to interrupt coronary blood flow. Without cardioprotective strategies such as cooling and chemical arrest, the heart would soon die. Unfortunately, no cardioprotective strategy has been shown to be optimal and some degree of post CPB contractile dysfunction is inevitable. This is not only a problem in the adult patient undergoing

coronary artery bypass surgery (CABG) or other surgical procedures, it is also a significant clinical problem during surgical heart procedures to correct congenital heart defects in neonates.

[0049] Thus, local administration of the agent can begin at any time once surgery begins until twenty-four hours after surgery has ended. More typically, within 12 hours of surgery ending. Any range within these ranges can be used, such as 1, 2, 3, 4, or more hours after surgery has ended.

[0050] In certain embodiments, administration of the agent can begin before surgery, for example using a percutaneous approach for delivery of the agent.

[0051] Accordingly, the methods of the present invention can be used to treat any subject while coupled to a CPB circuit, i.e. during cardiac surgery, and/or following cardiac surgery, during their transition off of the CPB circuit. Cardiac surgery includes any surgical procedure on the heart and usually involves interruption of coronary blood flow. It can also be used to assist the heart function during and after any thoracic surgical procedure where the heart is already exposed to the surgeon.

[0052] Before turning the CPB circuit, also known as the pump, off, all clinical determinants of cardiac performance are evaluated and adjusted, in order to optimize cardiac output. All metabolic, thermal, electrolyte, acid/base, and hematologic abnormalities are corrected. Blood volume is adjusted according to central venous, left atrial or pulmonary artery pressures. Peripheral resistance is estimated and vasodilators or constrictors are instituted as required. After the drug's effectiveness is assessed, pump flow is decreased in small increments while venous return to the heart is proportionately adjusted to maintain a constant filling pressure by constricting the venous drainage to the CPB circuit.

[0053] The assessment of cardiac function by transesophageal echocardiography and hemodynamic data immediately before terminating CPB allows

patients to be classified into 3 groups by decreasing risk, referred to herein as groups A, B, and C [Souza et al., Indian Journal of Extracorporeal Technology 6:2, 1998]. The methods of the present invention can be used to treat any patient in group A, B, or C, including children in need of inotropic support during cardiac surgery or during weaning from CPB.

[0054] The highest risk patients, classified herein as "Group A" patients, have severe cardiac dysfunction that makes it difficult to be removed from CPB, despite physiologic and pharmacological support. For these patients CPB is prolonged. Group A patients are by definition the hardest cases to manage. A few of these patients by the end of rewarming of the blood will have minimal or no cardiac activity, which precludes any trial of disconnection from pump. The remaining patients may be given a short trial off pump after optimization of preload, afterload and contractility by a combination of inotropes and vasoactive agents. Some of these patients will tolerate CPB removal, under maximal physiological and pharmacological support, and a few in the group may be further improved by an intra-aortic balloon pump. The patients with minimal cardiac activity and those in whom the trial off pump was unsuccessful are temporarily maintained on cardiac support with the heart-lung machine. A few hours on pump support may be a sufficient rest period to allow recovery of cardiac function and removal of CPB support in a small number of cases. For the others, a decision has to be made as to either advance to a mechanical device for prolonged support or terminate the efforts to recover cardiac action.

[0055] Children in Group A supported by full veno-arterial extracorporeal membrane oxygenation (ECMO) post cardiotomy have a poor long term survival rate [Langley et al., Eur J Cardiothorac Surg 13, 520-5, 1998] when compared with children managed with centrifugal ventricular assist devices [Thuys et al., Eur J Cardiothorac Surg 13, 130-4, 1998]. The methods of the present invention may be utilized in the treatment of children in Group A.

[0056] In certain cases, a few hours of circulatory assistance and intensive inotropic and vasodilator drug therapy may turn some Group A patients into group B. The remaining patients are candidates to a form of total circulatory mechanical support (if available) or they will not likely survive disconnection from pump [Harris C. et al., Tecnol. Extracorp. Rev. Latinoamer. 3, 13-19, 1996; El-Banayosy A., et al., Perfusion, 11, 93-102, 1996; Núñez HI., Tecnol. Extracorp. Rev. Latinoamer. 2, 33-41, 1995].

[0057] Group B patients have a mild to moderate degree of cardiac dysfunction, and require greater support and a more elaborate protocol for CPB termination than patients in Group C. Final preparations are made on partial bypass. In addition to the delivery of inotropic agents using the present invention, these patients may also be supported by physiological means such as volume resuscitation or additional pharmacological means, namely vasodilators. Some patients in this group can benefit from intra-aortic balloon pumping. Patients in this group will benefit from the methods of the present invention.

[0058] Some Group B patients may have to return to pump for better adjustment of drugs, or to have an intra-aortic balloon inserted if a marginal cardiac output is present, as demonstrated by atrial and arterial pressures, arterial and venous blood gases and pH, and spontaneous diuresis.

[0059] Group B patients include children with preoperative intracardiac shunts leading to high pulmonary blood flow, children after a heart transplant, and some adults with long standing congestive heart failure, who may present with pulmonary hypertension that precludes successful weaning. In certain instances, inhalation of nitric oxide (NO) can improve pulmonary hypertension and cardiac output and support discontinuance of CPB. Additional Group B patients include patients who received inadequate myocardial protection for any reason, including inadequate re-dosing of cardioplegia, inadequate perfusion of myocardia with

cardioplegia, patients with severe ventricular hypertrophy or aortic insufficiency, surgical errors, and prolonged CPB time.

[0060] An occasional patient in group B will not tolerate CPB termination even after a few trials. These few exceptions turn into group A patients.

[0061] For lower risk "Group C" patients, inotropic support of the present invention can be provided at a lower level, and may be discontinued as the patient arrives at the intensive care area or a few hours thereafter. The methods of the present invention can be used as needed to treat Group C patients, who are anticipated to smoothly disconnect from perfusion. For these patients, after reestablishing ventilation to the lungs, pump flow can be gradually reduced while venous return to the oxygenator is decreased until bypass is minimal. Arterial pump is stopped and venous line is clamped. Final adjustment of cardiac performance is made off pump, by slowly administering residual volume from the oxygenator until ideal preload is attained. These patients maintain an adequate cardiac output, as can be confirmed by normal atrial and arterial pressures, arterial and venous blood gases and pH and adequate spontaneous diuresis.

[0062] In one particularly preferred embodiment of the invention, the methods can be used to treat any subject undergoing non cardiac thoracic surgery where the heart is exposed, to assist the heart function and/or to treat contractile dysfunction.

[0063] In some embodiments, the inotropic agent of the present invention can be co-administered with prostaglandin E1, which can act as a powerful adjunct to wean difficult transplanted children with right ventricular failure.

[0064] In some embodiments, the inotropic agent of the present invention can be co-administered with nitroprusside or other vasodilator drugs.

[0065] In some embodiments, one particularly preferred inotrope is enoximone, to provide pharmacological support during weaning of patients with severe ventricular dysfunction.

[0066] The term "subject" as used herein refers to vertebrates, particularly members of the mammalian species and includes but is not limited to, domestic animals, sports animals, primates, dogs, cats, rodents including mouse and rat, horse and humans; more preferably, the term refers to humans.

Inotropic Agents

[0067] As used herein, "inotropic agents" or "positive inotropic agents" or "inotropes" or "positive inotropes" or "inotropic antibodies" will be used interchangeably and refers to the effect such agents produce, i.e. improves cardiac output by increasing the force of myocardial muscle contraction. "Positive inotropic effect" means that the contractility of the cells is enhanced in a dose-dependent manner. A positive inotropic effect-producing amount of an inotropic agent of the invention can be administered to a subject.

[0068] Positive inotropic agents of the present invention include any agents which provide the heart with contractile support. The agent can be an inotropic agent such as a sympathomimetic or a phosphodiesterase inhibitor, as long as one obtains the desired contractile effect on the heart. Inotropic compounds include agents that interact with the sympathetic nervous system and modulate calcium entry, G-proteins, ATP and GTP. Inotropic compounds include sympathomimetic compounds, phosphodiesterase inhibitors, BNP, ANP, and digitalis glycosides. Preferably, the agent is a sympathomimetic or a phosphodiesterase inhibitor. Preferred sympathomimetics include but are not limited to epinephrine, norepinephrine, dopamine, dobutamine, dopexamine, terbutaline, and isoproterenol, and analogues and derivatives thereof. Preferred phosphdiesterase inhibitors include but are not limited to milrinone, amrinone, enoximone, and pimobendan, and analogues and derivatives thereof.

[0069] Preferably, the positive inotropic agent is administered in the form of a pharmaceutical composition. A pharmaceutical composition comprising an effective amount of the positive inotropic agent as an active ingredient can be prepared by standard procedures well known in the art, with pharmaceutically acceptable non-toxic solvents and/or sterile carriers, if necessary. For example, the inotropic agent can be embedded in a controlled-release polymer. In other embodiments the positive inotropic agent is administered without a pharmaceutical carrier.

[0070] The dose of the positive inotropic agent is a therapeutically effective dose. In particular embodiments, the positive inotropic agent can be administered at a dose which produces in the subject an effect equivalent to the systemic intravenous administration of between 2 and 20 mcg/kg/min. However, in other embodiments, higher and lower dosages can be administered to subjects. For example, a dose which produces in the subject an effect equivalent to the systemic intravenous administration of 0.5 mcg/kg/min, or 40 mcg/kg/min. Optimizing therapy to be effective across a broad population can be performed with a careful understanding of various factors to determine the appropriate therapeutic dose. Typically, the dose can be much lower than the dose administered by intravenous infusion, because the agent is being locally delivered to the heart, rather than systemic administration.

Localization of the Delivery Vehicle on the Heart

[0071] Routes for direct application of the delivery vehicle to the heart include any routes which allow the delivery vehicle to be applied locally to the heart. For example, the delivery vehicle may be applied from the blood stream, by being placed directly in the heart through the coronary arteries or veins onto the heart surface; or through the ventricular or atrial walls and onto the heart surface. The delivery vehicle may also be applied through direct application during extensive surgical field exposure, or through direct application during minimally invasive exposure, for example through a pericardial window or heart port. The delivery

vehicle may also be applied through a percutaneous route, or via endovascular catheters.

[0072] In one embodiment, the delivery vehicle is loaded with the inotropic agent and placed over the heart of a surgical patient, before the sternum is closed, allowing direct release of the inotropic agent to the heart.

[0073] Placement of the delivery vehicle can be understood with reference to the different compartments of the heart. The heart is subdivided by a muscular septum into two lateral halves, which are named respectively right and left. A transverse constriction subdivides each half of the heart into two cavities, or chambers. The upper chambers consist of the left and right atria, which collect blood and help fill the lower chambers. The lower chambers consist of the left and right ventricles, which pump blood to the rest of the body. The chambers are defined by the epicardial wall of the heart. The right atrium communicates with the right ventricle by the tricuspid valve. The left atrium communicates with the left ventricle by the mitral valve. The right ventricle empties into the pulmonary artery by way of the pulmonary valve. The left ventricle empties into the aorta by way of the aortic valve.

[0074] The circulation of the heart consists of two components. First is the functional circulation of the heart, i.e., the blood flow through the heart from which blood is pumped to the lungs and the body in general. Second is the coronary circulation, i.e., the blood supply to the structures and muscles of the heart itself. The functional circulation of the heart pumps blood to the body in general, i.e., the systemic circulation, and to the lungs for oxygenation, i.e., the pulmonic and pulmonary circulation. The left side of the heart supplies the systemic circulation. The right side of the heart supplies the lungs with blood for oxygenation. Deoxygenated blood from the systematic circulation is returned to the heart and is supplied to the right atrium by the superior and inferior venae cavae. The heart pumps the deoxygenated blood into the lungs for oxygenation by way of the main pulmonary

artery. The main pulmonary artery separates into the right and left pulmonary arteries, which circulate to the right and left lungs, respectively. Oxygenated blood returns to the heart at the left atrium via four pulmonary veins. The blood then flows to the left ventricle where it is pumped into the aorta, which supplies the body with oxygenated blood.

[0075] The functional circulation supplies blood to the heart by the coronary circulation. The coronary arteries arise from the proximal aorta through the left and right coronary ostia course along the epicardial surface of the heart and send of numerous branches to supply the myocardium. Blood is cleared from the muscle by cardiac veins that flow into the coronary sinus and right atria. The heart wall is surrounded by a pericardial sac, which contains it within interstitial fluid.

[0076] In one embodiment, the delivery vehicle loaded with the inotropic agent is placed over the heart, before the sternum is closed, allowing direct release of the inotropic agent to the heart. In one embodiment, the delivery vehicle is placed away from the sinoatrial node or the right atrium. A preferred placement of the delivery vehicle is on the apex of the ventricle or left ventricular free wall.

[0077] Another embodiment of the invention provides the use of a non-permeable barrier on the surfaces of the heart not treated with the delivery vehicle, to achieve additional localization. In another embodiment, the delivery vehicle itself can be coated with a non-permeable barrier, to further localize release of the agent directly to the underlying heart tissue, while minimizing release into the pericardial fluid.

[0078] One particularly preferred embodiment provides local delivery of dopamine, epinephrine, norepinephrine, isoproterenol, and dobutamine to the ventricle without targeting the sinus node in the right atrium, limiting the excessive tachycardia observed in intravenous infusion.

[0079] In one embodiment, the delivery vehicle contains an inotropic agent that must be activated or released by a second agent. That second agent can be added

systemically to locally activate or release the inotropic agent. In this way, timing and/or release can be controlled at later points.

Treatment Period

[0080] Preferably, the delivery methods of the present invention are administered to the subject just long enough to support the heart until it recovers from its weakened condition. The short term or transient administration of the inotropic agent may last for a period of several minutes to several days. For example, from five minutes to 14 days. Typically, at least two hours to seven days. Preferably five hours to five days. More preferably, 2-24 hours. One can use all ranges between 5 minutes to 14 days, e.g. 12 hours to 12, 11, 10, 9, 8, 7, or fewer days.

[0081] In one embodiment of the invention, the patient is a surgical patient and the delivery methods of the present invention can be used to treat the heart prior to surgery, during surgery, after surgery, and any combination thereof.

Delivery Vehicle

[0082] The delivery vehicle of the present invention is any drug delivery means that can incorporate an inotropic agent, and is suitable for administration directly to the heart for local delivery or release of that agent. Suitability for local delivery to the heart includes the ability of a delivery vehicle to adhere to the underlying tissue. Any delivery vehicle which can be loaded with an inotropic agent and locally applied to the heart can be used in the present invention.

[0083] Examples of delivery vehicles include but are not limited to a patch, a matrix, a hydrogel, a sheet of material, a foam, a gel, a cream, a spray, and an ointment. Certain preferred delivery vehicles are polymeric controlled release vehicles. In one embodiment, the delivery vehicle is a patch, such as a transepicardial patch, that slowly releases the agents directly into the myocardium. In one embodiment, the delivery vehicle is an ointment or cream which may be placed manually on the target area of the heart. In one preferred embodiment, the delivery

vehicle is a hydrogel, which may be polymerized either directly on the heart in vivo or polymerized in vitro to form a patch for administration.

[0084] In one preferred embodiment, the inotropic agent(s) of the invention are incorporated into a biocompatible delivery vehicle referred to as a matrix. The matrix can be in the form of a gel, foam, suspension, microcapsules, solid polymeric support, or fibrous structure. The matrix may also serve in a physically supporting role. There is no specific requirement as to thickness, size or shape. It is preferred that the matrix be sufficiently porous to allow the inotropic agent to diffuse out of the matrix into the surrounding tissue in roughly physiologic quantities.

[0085] Preferably, the matrix is a biodegradable material. Preferably, the hydrogel matrix degrades in a period of time minimizing tissue inflammation, for example in less than seven to ten days. Examples of a biodegradable matrices include but are not limited to synthetic polymers degrading by hydrolysis, for example, polyhydroxy acids like polylactic acid, polyglycolic acid and copolymers thereof, polyorthoesters, polyanhydrides, proteins such as gelatin and collagen, or carbohydrates or polysaccharides such as cellulose and derivatized celluloses, chitosan, alginate, or combinations thereof, so that over the course of several days or weeks after implantation of the matrix material, the matrix gradually disappears.

[0086] The use of biodegradable matrices eliminates the need for surgery to remove undegraded implanted matrix. However, synthetic non-biodegradable matrices may also be used. Useful materials include but are not limited to ethylene vinyl acetate, polyvinyl alcohol, silicone, polyurethane, non-biodegradable polyesters, and polyethyleneoxide-polypropyleneoxide, and tetrafluoroethylene meshes (Teflon®).

[0087] In a preferred embodiment, the matrix is a hydrogel, defined as a matrix wherein typically approximately 900-fold by weight of the matrix is absorbed water. Hydrogels are well known in the art. Hydrogels can be formed by ionic or covalent crosslinking of a variety of water soluble polymers such as

polyphosphazenes, polysaccharides such as alginate, and proteins such as gelatin. For example, one matrix material is purified gelatin-based Gelfoam™ (The Upjohn Co., Kalamazoo, Mich.) surgical sponge.

[0088] To achieve the above properties, the hydrogel is formed primarily of polymerized macromers, the macromers being themselves polymers or copolymers of one or more monomers having reactive groups providing resorbable linkages and polymerizable sites for biodegradability and polymerization. The macromers have sufficient hydrophilic character to form water-absorbent polymerized gel structures, and are at least dispersible in a substantially aqueous solution, and preferably are water-soluble, to maximize tissue adherence. The macromers are preferably made predominantly of synthetic materials. The resulting hydrogels are preferably highly compliant, so as not to impede the process of cardiac contraction. The hydrogels are preferably covalently crosslinked to ensure that they are retained at the site of application until the hydrogels degrade. In certain embodiments, the gel can be crosslinked *in situ*, for example by photopolymerization.

[0089] Monomers and macromers which are suitable for forming the hydrogels ("referred to here in this section collectively as "monomers") have one or more of the following properties: water soluble, partially macromeric character, containing hydrophilic groups, and being covalently reactive. When crosslinked to form gels, the resulting gels are tissue adhesive, elastic, and compliant. The monomers are preferably water soluble. Water soluble materials are soluble to at least about 0.1 gram per liter of a substantially aqueous solvent. A substantially aqueous solvent comprises at least about 50% by weight of water, and less than about 50% by weight of a non-aqueous, water-miscible solvent. If the polymers are not entirely water-soluble, they should be dispersible in water, and form micelles, typically with the aid of non-aqueous, water-miscible solvents. The non-aqueous solvent must be present in an amount that does not damage the tissue. Thus only a small amount of non-aqueous, water-miscible solvent should be present in the pre-gelled composition to minimize tissue irritation. Up to about 10% by weight of the solution can be a non-

aqueous, water-miscible solvent. Examples of non-aqueous, water-miscible solvents include ethanol, isopropanol, N-methylpyrrolidone, propylene glycol, glycerol, low molecular weight polyethylene glycol, DMSO, Benzyl alcohol, and benzyl benzoate. Liquid surfactants, such as poloxamers (e.g., PLURONIC™ surfactants) and some polyethylene glycol derivatives (e.g., some TWEEN™ surfactants) can also be used as non-aqueous, water-miscible solvents.

[0090] The monomers are preferably at least partially macromeric, and are more preferably substantially to completely macromeric. Macromers tend to be innocuous to tissue because they will not readily diffuse into or penetrate cells. A macromer is a reactive monomer consisting of a polymeric material with a number-average or weight-average molecular weight of about 500 Daltons or more and at least one reactive group. To form a crosslinked gel by chain-growth polymerization, the macromers, along with any other smaller monomers, in a solution must contain on average more than one reactive group (which may be a covalently reactive group, or a group that binds non-covalently to other macromers). For polymerizations involving step-growth polymerization, the macromers must contain on average more than two reactive groups, and the solution typically contain approximately equal numbers of the two different types of reactive groups. An example of step-growth polymerization is gelation by formation of urethane linkages from the reaction of isocyanate with the hydroxyl groups. For free-radical polymerization of unsaturated materials (chain-growth polymerization), the monomers must contain on average more than one reactive group to crosslink.

[0091] The monomers are preferably covalently reactive, and thus form a covalently crosslinked gel. The crosslinked gels are elastic, and further are both elastic and compliant with soft tissue at low polymer concentrations.

[0092] Any method of covalent polymerization is potentially useful in the formation of the gels. The reactive groups may include, without limitation, ethylenically unsaturated groups, isocyanates, hydroxyls and other urethane-forming

groups, epoxides or oxiranes, sulfhydryls, succinimides, maleimides, amines, thiols, carboxylic acids and activated carboxylgroups, sulfonic acids and phosphate groups. Ethylenically unsaturated groups include acrylates and other unsaturated carboxylic acids, vinylic and allylic groups, cinnamates, and styrenes. Activated carboxyl groups include anhydrides, carbonylimidazoles, succinimides, carbonyl nitrophenols, thioesters, O-acyl ureas, and other conjugated carbonyls. In general, any reactive group that will covalently bond to a second and that can maintain fluidity when exposed to water for enough time to allow deposition and reaction is of use in making a suitable reactive macromer. Due to their excellent stability and slow reactivity in aqueous solutions, ethylenically unsaturated reactive groups are preferred.

[0093] The polymerization reaction does not have to result in covalent bonds. A number of materials are known which can form gel structures by changing the ionic conditions of the medium (e.g. alginate) or by changing the temperature of the medium (e.g., agarose, certain poloxamers). Polysaccharides are typical of these materials. Gel-like structures can be formed from proteins, such as gelatin or fibrin. While it maybe more difficult to get these materials to adhere strongly to tissue, they are potentially of use in the hydrogels, particularly as depots for the drug.

[0094] Gel formation can be accelerated by inclusion of small (non-macromeric) polymerizable molecules that can assist in linking larger, polymeric macromers. These typically have molecular weights less than about 100 Da, more preferably less than 500 Da. For free radical polymerization, any of the common ethylenically unsaturated molecules can be used. These include derivatives of acrylic and methacrylic acid, such as acrylamide, hydroxyethyl methacrylate (HEMA), and diacrylated or polyacrylated glycols and oligoglycols. Allyl groups (e.g., allyl glycidyl ether) and vinyl groups (e.g., N-vinyl caprolactam and N-vinyl pyrrolidone) are also of use. Other unsaturated compounds include cinnamic acid and its esters, and maleic, fumaric and itaconic acids and their derivatives.

[0095] Polymerization is initiated by any convenient reaction, including photopolymerization, chemical or thermal free-radical polymerization, redox reactions, cationic polymerization, and chemical reaction of active groups (such as isocyanates, for example.) Polymerization is preferably initiated using photoinitiators. Photoinitiators that generate a free radical or a cation on exposure to UV light are well known to those of skill in the art. Free-radicals can also be formed in a relatively mild manner from photon absorption of certain dyes and chemical compounds. The polymerizable groups are preferably polymerizable by free radical polymerization. The preferred polymerizable groups are acrylates, diacrylates, oligoacrylates, methacrylates, dimethacrylates, oligomethacrylates, cinnamates, dicinnamates, oligocinnamates, and other biologically acceptable photopolymerizable groups.

[0096] These groups can be polymerized using photoinitiators that generate free radicals upon exposure to light, including UV (ultraviolet) and IR (infrared) light, preferably long-wavelength ultraviolet light (LWUV) or visible light. LWUV and visible light are preferred because they cause less damage to tissue and other biological materials than short-wave UV light. Useful photoinitiators are those which can be used to initiate polymerization of the macromers without cytotoxicity and within a short time frame, minutes at most and most preferably seconds. Exposure of dyes, preferably in combination with co-catalysts such as amine, to light, preferably visible or LWUV light, can generate free radicals. Light absorption by the dye causes the dye to assume a triplet state, and the triplet state subsequently reacts with the amine to form a free radical which initiates polymerization, either directly or via a suitable electron transfer reagent or co-catalyst, such as an amine. Polymerization can be initiated by irradiation with light at a wavelength of between about 200-1200 nm, most preferably in the long wavelength ultraviolet range or visible range, 320 nm or higher, and most preferably between about 365 and 550 nm. Numerous dyes can be used for photopolymerization. Suitable dyes are well known to those of skill in the art. Alternatively, suitable chemical, thermal and redox systems may initiate the polymerization of unsaturated groups by generation of free radicals in the initiator

molecules, followed by transfer of these free radicals to the unsaturated groups to initiate a chain reaction. Examples include but are not limited to peroxides, other peroxygen compounds, and azobisbutyronitrile.

[0097] As used herein, a "biodegradable" material is one that decomposes under normal in vivo physiological conditions into components that can be metabolized or excreted. Functional groups having degradable or resorbable linkages are incorporated into the structure of the hydrogel matrix to provide for its resorption over time. These functional groups may be incorporated within the macromers to form part of the backbone of the polymer strands of the hydrogel or as crosslinks between the polymer strands. Examples of degradable units may include, but are not limited to, esters, carbonates, carbamates and the like. The length of time it takes for the hydrogel to biodegrade may be tailored to provide a hydrogel that persists long enough to generate the required tissue level of the drug through the treatment period, which can last up to the seventh postoperative day, or preferably up to the tenth or fourteenth day. Given the achievement of this objective, shorter degradation or resorption times such as less than about three months are generally preferred. Degradation or resorption times less than about fifteen days are particularly preferred.

[0098] As used herein, a "biocompatible" material is one that stimulates only a mild, often transient, implantation response, as opposed to a severe or escalating response. Biocompatibility may be determined by histological examination of the implant site at various times after implantation. One sign of poor biocompatibility can be a severe, chronic, unresolved phagocytic response at the site. Another sign of poor biocompatibility can be necrosis or regression of tissue at the site. In the preferred embodiment, a biocompatible material elicits a minimal or no fibrosis or inflammation. This can be achieved through selection of hydrogel composition, and particularly through the use of hydrogel components resulting in degradation of the hydrogel in vivo in less than about two weeks, more preferably within seven to ten days.

[0099] In a preferred embodiment, the hydrogel composition is selected to provide acceptable levels of fibrosis or tissue reaction. This can be achieved through the selection of the reactive formulation, and other techniques known to those skilled in the art in drug delivery utilizing polymeric delivery devices.

[00100] Preferably, the inotropic agents are poorly soluble in water (i.e. hydrophobic). In terms of the solubility classification of the United States Pharmacopoeia (USP 24/NF 19, effective Jan. 1, 2000; p. 2254), the preferred solubility classes are: "slightly soluble", requiring 100 to 1000 parts of solvent to dissolve; "very slightly soluble", requiring 1000 to 10,000 parts of solvent; and "practically insoluble, or insoluble", requiring over 10,000 parts of solvent. Collectively, these classes are defined herein as "poorly soluble".

[00101] An inotropic agent applied in a single application directly to the heart is expected to be similarly or more effective to intravenous administration, with a potential reduction in side effects because a lower required dose and limited spread is anticipated.

[00102] The slow dissolution rate for poorly soluble inotropic agents controls their rate of efflux from the gel. The rate of efflux for such inotropic agents can also be controlled by selecting the particle size of the drug particles that are suspended in the macromer solution before its polymerization. Particles of a particular size can be made by any known method, including grinding, milling, cryofracture, precipitation, spraying, spray drying, and/or classification. Dispersion and stabilization of the particles within the macromer solution may be achieved with the use of surfactants.

[00103] When more soluble inotropic agents are used, their efflux rate from the gel can be altered to achieve the necessary delivery rate. Such soluble inotropic agents include those falling in United States Pharmacopoeia classes "very soluble", "freely soluble", "soluble", and "sparingly soluble". Typical means of altering release rates include encapsulating the agents in micro particles or liposomes and conjugating

the agents to macromolecules. They can be made less soluble by altering the salt or using the free acid/base form of the agents.

[00104] In one embodiment, pre-encapsulation is used for the small, water-soluble drugs (typically of molecular weights less than 1000 Da) that are incorporated into hydrogels, to decrease the rate of release of these drugs. The encapsulation may be by any conventional means. One means is entrapment in micro particles of a degradable, water-insoluble polymer. Typical materials are polymers and copolymers of lactic acid, glycolic acid, and copolymers thereof (e.g., PLGA). Other materials used to form suitable micro particles are copolymers of ethylene and vinyl acetate (EVAC) and polymers of anhydrides, such as poly sebacic anhydride. Particles of drug may also be pre-encapsulated with polymers such as EVAC and PLGA, or with thin layers of materials that dissolve in vivo, for example, the enteric coatings or other coatings typically used for oral delivery, such as gelatin.

[00105] Release of more soluble inotropic agents can be slowed by conjugating small molecules to polymers by degradable or reversible linkages. Many such systems are described in the art. In one embodiment, such systems are generated by immobilizing a binding or targeting molecule for the drug, such as an antibody or lectin, which is saturated with the drug, in the gel. In another typical embodiment, drug is attached to a polymer bearing reactive groups, such as to the hydroxyl of polyvinyl alcohol, to a carboxyl, sulfonate or amine group of a polysaccharide or the hydroxyl or carboxyl of an alpha-hydroxy acid (e.g., lactic or glycolic acid), or to a carboxylic group on a polymer (e.g., alginate, polyacrylic acid) via an anhydride, an ester, a carbonate, or carbamate linkage. Many similar methods are described in the art.

[00106] The solubility of some agents can be decreased by preparing them in their neutral ("free base") form. Such agents often can also be administered as suspensions in oil, which in turn is dispersed in water, usually with surfactant stabilizers.

[00107] The level of loading of the inotropic agent in the delivery vehicle will normally be as high as practical, while leaving a margin of loading to prevent premature precipitation or aggregation, or inhibition of gel formation. The concentration of the inotropic agent can be between 0.5 and 1% by weight, but this will depend in part upon the source and form of the inotropic agent. Gel polymerization rate and final gel may be significantly affected by drug concentration. Use of other macromers affects the optimal level. Fortunately, acceptable loading ranges are easily determined for a particular system by varying the loading and determining the properties of the formed gel.

[00108] In one method, the inotropic agent is provided in a formulation that forms a hydrogel in vivo, i.e. after its components are administered to the heart.

[00109] In a second method, the inotropic agent is provided to the patient in a preformed hydrogel "patch", i.e. formed before administration to the heart.

[00110] The hydrogels of the present invention are formed by a polymerization reaction, which may be any reaction that can be carried out in a substantially aqueous environment and is not damaging to tissue. The gels may be polymerized in vivo or in vitro.

[00111] The adherence of gels to tissue can be optimized by techniques that employ functional primers, as described in U.S. Pat. No. 5,800,373 to Melanson et al., U.S. Pat. Nos. 5,844,016, or 5,900,245 to Sawhney et al. for gels formed by polymerization of ethylenically unsaturated precursors. Suitable gel compositions form strong bonds to tissue. These techniques are also applicable to creating strong adherence of the materials to tissue, including tissue to which it is difficult to obtain adherence by conventional methods, for example, cartilage.

[00112] A general procedure for applying materials to the tissue involves brushing or dabbing primer over a larger area than that over which the material is applied. Thereafter, material is brushed or dabbed over the deposited primer. Then

bulk material is applied by dripping (if liquid) or spreading (if paste) over yet a smaller area of the treated zone. Then light (at appropriate wavelength, intensity, distance and for an appropriate time) is applied at each zone, or other means of polymerizing the material are used.

[00113] Methods for in vivo and in vitro hydrogel polymerization are known in the art, for example as described in published patent applications 20020150622 and 20050004428, which are hereby incorporated by reference.

[00114] For in vivo polymerization, the inotropic agent is formulated in appropriate excipients (if any) in a vial, and is taken up in a known amount of hydrogel forming material. This solution is applied to the tissue, and polymerization is effected to form a gel adherent to the tissue. Preferably, the solution is polymerized by illumination of a photoinitiator or photosensitizer in the solution. In this case, the mixing of two solutions at the time of application will not necessarily form a gel; however once the solutions are illuminated by light of an appropriate frequency, a gel will form, as described in U.S. Pat. No. 5,410,016 to Hubbell et al. incorporated herein by reference in its entirety.

[00115] In vivo polymerization has the advantage of being able to produce "good" to "excellent" adherence when polymerized on the tissue surface. This is particularly true when the tissue is first primed or otherwise pretreated with an agent (primer) stimulating polymerization (as known to those skilled in the art, for example, as described in U.S. Pat. No. 5,844,016 to Sawhney et al. and U.S. Pat. No. 5,834,274 to Hubbell et al. incorporated herein by reference in their entirety) prior to the application of the macromer composition containing the inotropic drug. See also U.S. Pat. Nos. 5,567,435; 5,844,016; 5,986,043; 6,060,582; and 6,306,922 incorporated herein by reference in their entirety. In these methods, an aqueous solution containing a photoinitiation system, including one or more photoinitiators, photosensitizers and co-initiators, amine or amide electron transfer agent, redox accelerant system for the photoinitiation system (such as a metal ion and a peroxide); and a photopolymerizable

macromer solution, are applied to the tissue, and the solution is polymerized by exposure to UV or visible light at room or body temperature.

[00116] For in vitro polymerization, hydrogel patches containing the inotropic agent are polymerized in vitro and then adhered to the surface of the heart. The inotropic agent in any suitable formulation can be entrapped in a hydrogel in vitro, which is optionally preserved by freezing or drying, and is subsequently transferred to the cardiac tissue. The preformed gel patch, or more than one preformed gel patch, is then adhered to the cardiac tissue. Adhesion of the patch may be achieved by the polymerization of a hydrogel-forming material, which may be the same as or different from the material used to form the gel patch, placed between the preformed gel patch and the tissue, or optionally encapsulates the entire pre-formed gel. Adhesion may also be achieved by completing polymerization of a partially polymerized gel patch onto the tissue. A partially polymerized gel patch is prepared by reducing time exposure to polymerization conditions or by quenching polymerization.

[00117] In vitro polymerization has the advantage of providing a reliable means of delivering a precisely defined dose of the inotropic agent. The preformed gels should have the same properties as gels formed in vivo. This method of application may be regarded as another form of application of an encapsulated drug to the tissue, since the adhesion to the tissue is provided by a hydrogel that is formed in situ on the tissue. The preferred method of attaching the gels to the tissue surface is to use macromer solutions to adhere the preformed gel to the tissue. Adherence is also preferably in the "good" to "excellent" range.

[00118] A material is tissue adherent if it requires a force to remove the material from the tissue. Thus, the general and practically useful measurement of adherence is that the gel, when applied to the tissue, remains attached to the tissue for at least as long as is required to obtain the therapeutic effect of the drug. Typically, this time period will be sufficiently long to observe at least about 10% elution of the

drug, and preferably 20% elution or more, before detachment or degradation of the gel.

[00119] Ex vivo tests can be used to determine a material's potential adherence. In evaluating potential adherence of materials, it is useful to have an in vitro test to determine formulations that are likely to have the desired degree of adherence to the tissue surface. One method of judging adherence is to require that upon a gradual increase in a detaching force, the force required to remove the gel from the tissue is greater than or approximately equal to the force required to cause cohesive failure of the gel (or the tissue, if lesser). Thus on attempting to remove the material, either the material or the tissue experiences cohesive failure at a lesser force than, or at approximately the same force as, the force at which the bond between the material and the tissue experiences adhesive failure. Materials that require a force of about 20 dynes/cm^2 to remove them from the tissue are sufficiently adhesive for delivery of inotropic agents.

[00120] Adherence can be described qualitatively as "excellent", when cohesive failure is required for removal from the surface, "good" when failure is partially cohesive and partially adhesive, "fair" when removal requires only adhesive failure (i.e., detachment of the gel from the surface) and more than 20 dynes/cm^2 of force is required to produce adhesive failure, and "poor" if none of these criteria are satisfied. Force can be measured using a mechanical properties tester, such as an Instron™ tester or other device.

[00121] The delivery vehicles of the invention are preferably highly compliant with the tissue to which they adhere. Thus, the delivery vehicles stretch and bend along with the tissue. Cardiac tissue is in continual motion, and the delivery vehicle should not significantly disturb this motion. It is preferable that the response to stress within these limits be substantially elastic, i.e., reversible. Thus the delivery vehicle should remain as a coherent material for at least the period required for delivery of the inotropic agent.

[00122] Techniques for producing strong adherence of a preformed hydrogel, a patch, or other delivery vehicle to the cardiac tissue include applying an initiator or promoter of polymerization to the tissue at the site; applying a thin layer of gelling solution having a high concentration of a polymerizable reagent at the site; applying materials bearing one half of a reactive pair to the site, optionally a member of a reactive pair which is also reactive with tissue; and applying mechanical action to a layer of polymerizable material on the tissue (before polymerization) to ensure that no layer of fluid, such as mucus or the like, separates the polymerizable material from the tissue.

[00123] As described herein, the delivery vehicles of the invention, including hydrogels, patches, ointments and creams, can be applied at the time of surgery and the drug delivered directly to the affected cardiac tissue. For a hydrogel polymerized in situ, the gel can be applied in open surgery by any method. In one embodiment, the delivery vehicle such as an ointment, cream, or gel is preferably brushed or sprayed onto the tissue surface for example by using a device designed for percutaneous use, but may be dripped from a mixing apparatus.

[00124] The therapeutic compositions of this invention are administered by local administration to the heart, as by application of a patch, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

[00125] It is important to provide a way for the physician to deliver a well-defined amount of the inotropic agent, so that the therapeutic effect can be obtained.

[00126] The dosage of the inotropic agents for use in a human or animal and the minimum duration can be determined with only routine experimentation in view of animal studies and the known drug kinetics, including half-life, solubility and other

readily ascertainable properties. The effective dosage can be determined from tissue concentrations and physiological effects over time in cardiac tissue of animals, after application of a known concentration of the drug in the delivery vehicle. Such animal studies are routine in determining dosage for any drug. The dosage of the inotropic agent will also be optimized based on the period of time over which delivery is to be obtained and the release rate from the delivery vehicle, as well as the degradation characteristics of the delivery vehicle, to deliver a therapeutically effective dose to the heart tissue.

[00127] The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered and timing depends on the subject to be treated, capacity of the subject's myocardium to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual.

[00128] Any formulation containing the active ingredients, which is suitable for the intended use, as are generally known to those of skill in the art, can be used. Suitable pharmaceutically acceptable carriers are known to those of skill in the art. The carrier must be pharmaceutically acceptable in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

[00129] As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable physiological effects.

[00130] In one embodiment, the inotropic agent may be administered in liposomes or microspheres or microparticles. Methods for preparing liposomes and microspheres for administration to a patient are well known to those of skill in the art. U.S. Pat. No. 4,789,734, the contents of which are hereby incorporated by reference,

describes methods for encapsulating biological materials in liposomes. Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids and lipids added, along with surfactants if required, and the material dialyzed or sonicated, as necessary. A review of known methods is provided by G. Gregoriadis, Chapter 14, "Liposomes," Drug Carriers in Biology and Medicine, pp. 287-341 (Academic Press, 1979).

[00131] Microspheres formed of polymers or proteins are well known to those skilled in the art, and can be tailored for direct administration to the heart using the delivery vehicles of the present invention. Suitable liposomes for targeting ischemic tissue are generally less than about 200 nanometers and are also typically unilamellar vesicles, as disclosed, for example, in U.S. Pat. No. 5,593,688 to Baldeschweiler, entitled "Liposomal targeting of ischemic tissue," the contents of which are hereby incorporated by reference.

[00132] Preferred microparticles are those prepared from biodegradable polymers, such as polyglycolide, polylactide and copolymers thereof. Those of skill in the art can readily determine an appropriate carrier system depending on various factors, including the desired rate of drug release and the desired dosage.

[00133] The formulations may further include one or more optional accessory ingredient(s) utilized in the art of pharmaceutical formulations, e.g., diluents, buffers, binders, surface active agents, thickeners, lubricants, suspending agents, preservatives (including antioxidants) and the like. The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation.

[00134] The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of

auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

[00135] The compositions of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

[00136] Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

[00137] As with the use of other pharmaceutical compositions, the individual patient can be monitored by various ways, including but not limited to invasive hemodynamic monitors, including arterial and central venous pressure monitoring; pulmonary artery catheters, which can include hemodilution cardiac output monitors, and/or continuous mixed venous oxygen saturation monitoring, in addition to pulmonary artery and pulmonary capillary wedge pressures; transesophageal or transthoracic echocardiography; and continuous electrocardiographic monitoring.

EXAMPLES

Methods

[00138] Sprague Dawley rats (900g – 1100g) were anesthetized with an intraperitoneal injection of ketamine and xylazine. The rat was laid on a heating pad and maintained euthermic with warming lights. A 24 gauge intravenous catheter was placed in the tail vein and Ringer's lactate solution (LR) was infused at 10 cc/hr. The trachea was exposed through a vertical incision and cannulated with a 16 gauge blunt cannula that served as an endotracheal tube. The tube was connected to a ventilator for control of ventilation and respiration. The respiratory rate was set to 60 breaths per minute, inspiratory to expiratory time was set to 1:2 and inspiratory flow was 2 liters per minute. A midline sternotomy was performed, the heart exposed and the pericardium resected. The right carotid artery was dissected clean of fascia and care was taken to preserve the adjacent vagus nerve. A polyethelene (PE-50) cannula was inserted via an arteriotomy into the carotid artery and advanced through the ascending aorta into the left ventricle. This ventricular cannula was connected to a high fidelity pressure transducer and digital data acquisition system to record hemodynamic measures. Heart rate (HR), left ventricular systolic blood pressure (SBP), and the maximum rate of change of blood pressure in the left ventricle during isovolemic contraction (dp/dt max) was all recorded. The dp/dt max is the gold standard index of myocardial contractility.

[00139] Following cannulation the rat was stabilized for 30 minutes. Five-second recordings of HR, SBP and dp/dt max were captured every 3 to 5 minutes . SBP and dp/dt max were averaged over all the beats captured in the 5-second interval. Dobutamine (313 mcg/ml), a potent beta agonist inotropic agent, was delivered to the left ventricular free wall through the sternotomy using an infusion pump connected to a 24 guage IV cannula that was suspended directly over the heart (4 mcg/min, 0.8 ml/hr). In this fashion, drug was administered directly to the heart and only in the area exposed by resected pericardium. After 30 minutes the pericardial application of dobutamine was terminated and hemodynamic measurements were recorded for an additional 30 minutes.

Results

[00140] The HR, SBP and contractile response (dp/dt max) to the pericardial application of dobutamine are shown in Figures 1-3.

[00141] This experiment demonstrates that dobutamine can be applied directly to the myocardial surface and exert positive inotropic effects without the systemic effects seen with systemic infusion. Contractility, as expressed by the maximum dp/dt of the left ventricular pressure during isovolemic contraction increased significantly and shortly after dobutamine was applied to the free surface of the heart (Figure 1). Dobutamine given in an intravenous infusion, in addition to increasing myocardial contractility and cardiac output, dilates smooth muscle through peripheral beta-receptors and leads to vasodilatation and reduction in systemic blood pressure. In this experiment, local pericardial dobutamine increased systemic blood pressure, likely from increased force of contraction and cardiac output in the presence of constant vascular tone (Figure 2). This suggests that the usual peripheral vasodilatory side effects of dobutamine infusion were eliminated by local application to the heart. Dobutamine is also a potent chronotrope and topical application with possible diffusion to the sino-atrial node, which normally functions as the pacemaker for the heart, increased heart rate (Figure 3). These data show that potent inotropic agents such as sympathomimetics and phosphodiesterase inhibitors can be locally applied to the heart and improve contractile function, while minimizing systemic side effects.

[00142] All references described herein are incorporated by reference in their entirety.

We claim:

1. A use of a cardiac inotropic agent in the treatment of a subject in need therof, comprising locally administering to the subject a therapeutically effective amount of at least one inotropic agent.

2. The use of claim 1, wherein the inotropic agent is an agent that interacts with the sympathetic nervous system and modulates calcium entry, G-proteins, ATP, or GTP, wherein the inotropic agent is selected from the group consisting of sympathomimetic compounds, phosphodiesterase inhibitors, BNP, ANP, and digitalis glycosides, and derivatives and analogues thereof.

3. The use of claim 1, wherein the inotropic agent is a sympathomimetic compound selected from the group consisting of epinephrine, norepinephrine, dobutamine, isoproterenol, salbutamol, salmeterol, terbutaline, phenylephrine, ephedrine, clonidine and dopamine, and derivatives and analogues thereof.

4. The use of claim 1, wherein the inotropic agent is a phosphodiesterase inhibitor selected from the group consisting of milrinone, enoximone and amrinone, and derivatives and analogues thereof.

5. The use of claim 1, wherein the subject is a surgical patient and is selected from the group consisting of a cardiac surgery patient, a thoracic surgery patient, and a general surgery patient.

6. The use of claim 1, wherein the subject is a cardiac surgery patient, and wherein the cardiac surgery patient is selected from the group consisting of a cardiac surgery patient requiring support from a cardiopulmonary bypass circuit and a cardiac patient not requiring support from a cardiopulmonary bypass circuit.

7. The use of claim 1, wherein the subject has a condition selected from the group consisting of trauma, shock, acute congestive heart failure and chronic congestive heart failure.

8. The use of claim 1, wherein the therapeutically effective amount of the inotropic agent is sufficient to effect myocardial contractility.

9. The use of claim 1, wherein the inotropic agent is delivered locally to the heart by its inclusion in a delivery vehicle.

10. The use of claim 1, wherein the inotropic agent is delivered locally to the heart by its inclusion in a delivery vehicle, and wherein the delivery vehicle is selected from the group consisting of a drug-impregnated, coated or relasing sheet, patch, matrix, hydrogel, foam, gel, cream, spray, microshpere, microcapsule, composite and an ointment.

11. The use of claim 1, wherein the local administration comprises administering said inotropic agent directly to the heart via an open surgical wound.

12. The use of claim 1, wherein the local administration comprises administering said inotropic agent directly to the heart percutaneously.

13. A use of an inotropic agent in a method of reducing postoperative complications of cardiopulmonary bypass (CPB) surgery in a subject comprising: locally administering to a subject in need thereof an effective amount of an inotropic agent in conjunction with said CPB surgery of said subject.

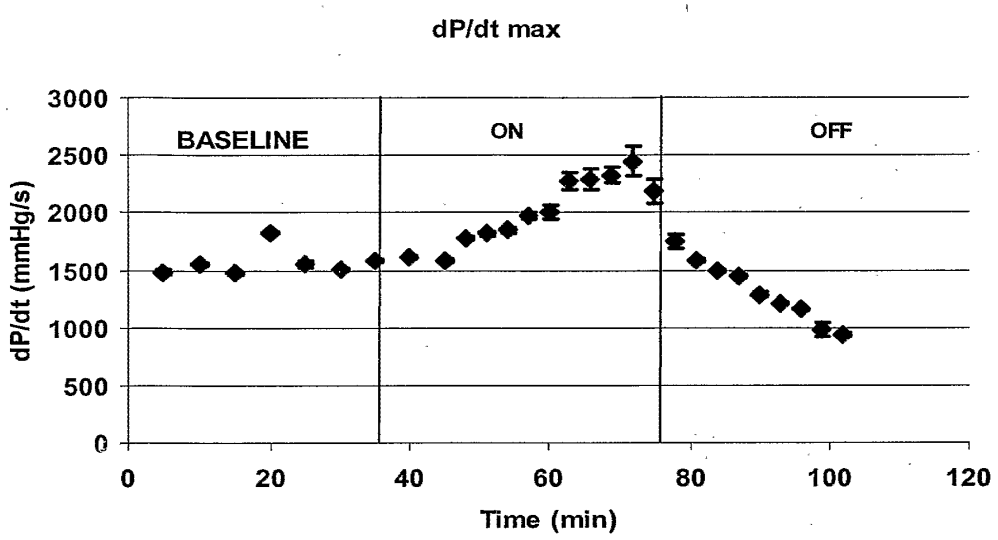
14. The use of claim 13, wherein the inotropic agent is administered to said subject during a time period selected from the group consisting of prior to said CPB surgery, during said CPB surgery, subsequent to said CPB surgery and combinations thereof.

15. The use of claim 13, wherein the inotropic agent is selected from the group consisting a sympathomimetic compound or a phosphodiesterase inhibitor.

16. The use of claim 13, wherein the therapeutically effective amount of the inotropic agent is sufficient to effect myocardial contractility.

17. The use of claim 13, wherein the inotropic agent is delivered locally to the heart by its inclusion in a delivery vehicle.

18. The use of claim 13, wherein the inotropic agent is delivered locally to the heart by its inclusion in a delivery vehicle, and wherein the delivery vehicle is selected from the group consisting of a drug-impregnated, coated or relasing sheet, patch, matrix, hydrogel, foam, gel, cream, spray, microshpere, microcapsule, composite and an ointment.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2500
2000
1500
1000
500
0
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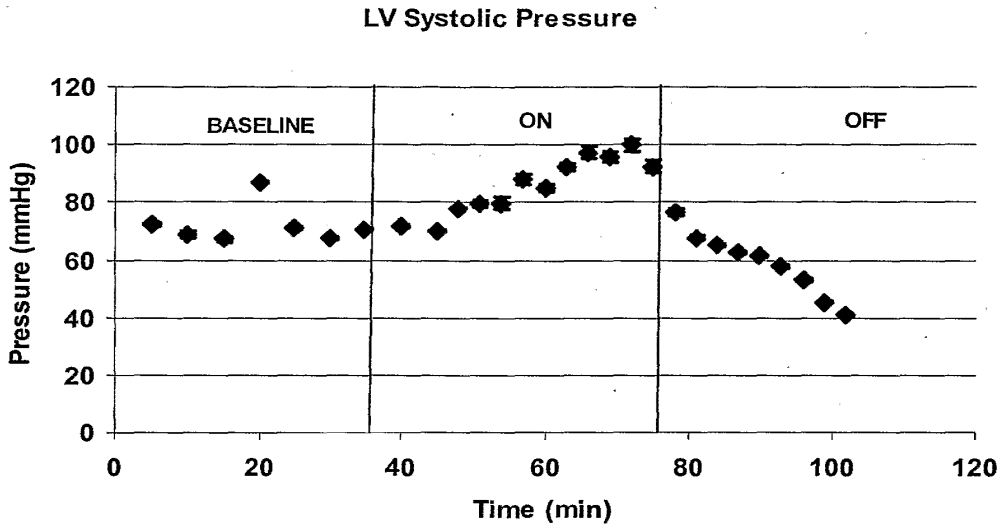
ON
OFF
0
20
40
60
80
100
120
Time (min)

FIGURE 1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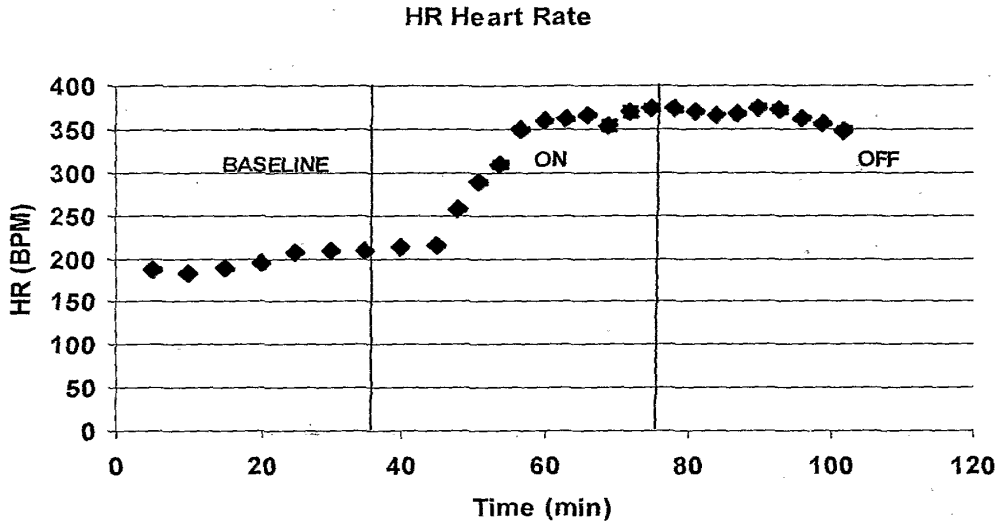
Time (min)

FIGURE 2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350
300
250
200
150
100
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HR (BPM)
BASELINE
ON
OFF
0
20
40
60
80
100
120
Time (min)

FIGURE 3

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TREATMENT OF PULMONARY ARTERIAL HYPERTENSION

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Abstract of WO 2010019540

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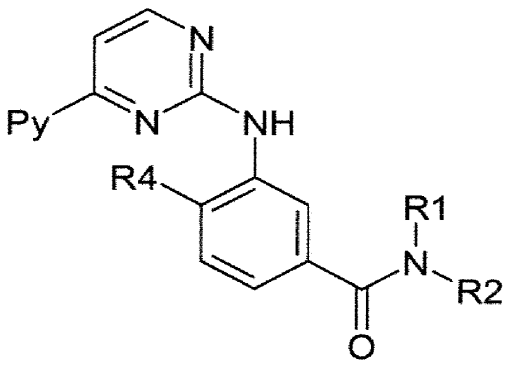
(54) **Title**: TREATMENT OF PULMONARY ARTERIAL HYPERTENSION



(I)

(57) **Abstract**: The present invention pertains to the use of 4-(4-methylpiperazin-1-ylmethyl)-N-[4-methyl-3-(4-pyridin-3-yl)pyrimidin-2-ylamino)phenyl]-benzamide or a pharmaceutically acceptable salt thereof or a pyrimidylaminobenzamide of formula I wherein the radicals and symbols are as defined herein, or a pharmaceutically acceptable salt thereof, for the manufacture of medicament for treating pulmonary arterial hypertension (PAH), especially in patients who failed prior PAH therapy.

WO 2010/019540 A1

TREATMENT OF PULMONARY ARTERIAL HYPERTENSION

The invention relates to the use of 4-(4-methylpiperazin-1-ylmethyl)-N-[4-methyl-3-(4-pyridin-3-yl)pyrimidin-2-ylamino)phenyl]-benzamide (also known as "Imatinib" [International Non-proprietary Name]; hereinafter: "COMPOUND I") or a pharmaceutically acceptable salt thereof or a pyrimidylaminobenzamide of formula I as defined below or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for the treatment of pulmonary arterial hypertension, to COMPOUND I or a pharmaceutically acceptable salt thereof or a pyrimidylaminobenzamide of formula I as defined below or a pharmaceutically acceptable salt thereof for the treatment of pulmonary arterial hypertension, and to a method of treating warm-blooded animals including humans suffering from pulmonary arterial hypertension, by administering to a said animal in need of such treatment an effective dose of COMPOUND I or a pyrimidylaminobenzamide of formula I or a pharmaceutically acceptable salt thereof.

Pulmonary arterial hypertension is a life-threatening disease characterized by a marked and sustained elevation of pulmonary artery pressure. The disease results in right ventricular (RV) failure and death. Current therapeutic approaches for the treatment of chronic pulmonary arterial hypertension mainly provide symptomatic relief, as well as some improvement of prognosis. Although postulated for all treatments, evidence for direct anti-proliferative effects of most approaches is missing. In addition, the use of most of the currently applied agents is hampered by either undesired side effects or inconvenient drug administration routes. Pathological changes of hypertensive pulmonary arteries include endothelial injury, proliferation and hyper-contraction of vascular smooth muscle cells (SMCs).

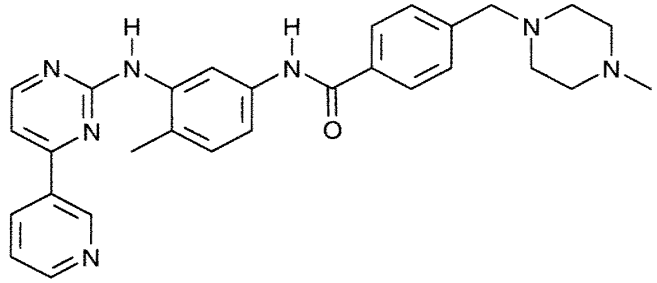
The instant invention is a response to the need for an alternative therapy in the treatment of pulmonary hypertension, especially pulmonary arterial hypertension.

United States patent specification US 2006/0154936 disclosed the use of COMPOUND I alone or in combination with other medication as an alternative to existing therapies for the treatment of pulmonary hypertension.

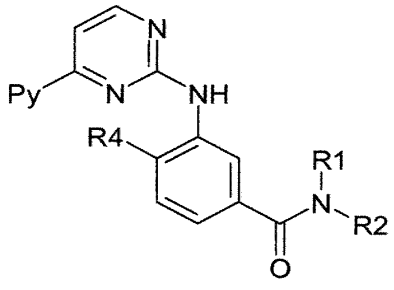
It has now surprisingly been demonstrated that pulmonary arterial hypertension can be successfully treated with COMPOUND I, or pharmaceutically acceptable salt thereof or a

pyrimidylaminobenzamide of formula I or a pharmaceutically acceptable salt thereof, in particular in patients who failed prior therapy.

In a first aspect the present invention concerns the use of COMPOUND I having the formula



or a pharmaceutically acceptable salt thereof, or a pyrimidylaminobenzamide of formula I



(I)

wherein

Py denotes 3-pyridyl,

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, acyloxy-lower alkyl, carboxy-lower alkyl, lower alkoxycarbonyl-lower alkyl, or phenyl-lower alkyl;

R_2 represents hydrogen, lower alkyl, optionally substituted by one or more identical or different radicals R_3, cycloalkyl, benzcycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted; and

R_3 represents hydroxy, lower alkoxy, acyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amino, mono- or disubstituted amino, cycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted;

or wherein R_1 and R_2 together represent alkylene with four, five or six carbon atoms optionally mono- or disubstituted by lower alkyl, cycloalkyl, heterocyclyl, phenyl, hydroxy, lower alkoxy, amino, mono- or disubstituted amino, oxo, pyridyl, pyrazinyl or pyrimidinyl; benzalkylene with four or five carbon atoms; oxaalkylene with one oxygen and three or four carbon atoms; or azaalkylene with one nitrogen and three or four carbon atoms wherein nitrogen is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, lower alkoxycarbonyl-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, N-mono- or N,N-disubstituted carbamoyl-lower alkyl, cycloalkyl, lower alkoxycarbonyl, carboxy, phenyl, substituted phenyl, pyridinyl, pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen, lower alkyl, or halogen;

or a pharmaceutically acceptable salt thereof, for the manufacture of a medicament for treating pulmonary arterial hypertension, especially in patients who failed prior PAH therapy.

In a second aspect the present invention concerns 4-(4-methylpiperazin-1-ylmethyl)-N-[4-methyl-3-(4-pyridin-3-yl)pyrimidin-2-ylamino)phenyl]-benzamide or a pharmaceutically acceptable salt thereof, or a pyrimidylaminobenzamide of formula I as defined above or a pharmaceutically acceptable salt thereof, for use in treating pulmonary arterial hypertension (PAH) in patients who failed prior PAH therapy.

In a third aspect the present invention concerns a method of treating warm-blooded animals including humans suffering from pulmonary arterial hypertension, by administering to a said animal in need of such treatment an effective dose of 4-(4-methylpiperazin-1-ylmethyl)-N-[4-methyl-3-(4-pyridin-3-yl)pyrimidin-2-ylamino)phenyl]-benzamide or a pharmaceutically acceptable salt thereof or a pyrimidylamino-benzamide of formula I as defined above or a pharmaceutically acceptable salt thereof.

In a fourth aspect the present invention concerns a method of treating a human suffering from

(a) idiopathic or primary pulmonary hypertension,

(b) familial hypertension,

(c) pulmonary hypertension secondary to, but not limited to, connective tissue disease, congenital heart defects (shunts), pulmonary fibrosis, portal hypertension, HIV infection, sickle cell disease, drugs and toxins (e.g., anorexigens, cocaine), chronic hypoxia, chronic pulmonary obstructive disease, sleep apnea, and schistosomiasis,

(d) pulmonary hypertension associated with significant venous or capillary involvement (pulmonary veno-occlusive disease, pulmonary capillary hemangiomatosis),

(e) secondary pulmonary hypertension that is out of proportion to the degree of left ventricular dysfunction,

(f) persistent pulmonary hypertension in newborn babies,

especially in patients who failed prior PAH therapy, which comprises administering to said human in need of such treatment a dose effective against the respective disorder of 4-methylpiperazin-1-ylmethyl)-N-[4-methyl-3-(4-pyridin-3-yl)pyrimidin-2-ylamino)phenyl]-benzamide or a pyrimidylaminobenzamide of formula I as defined above or a pharmaceutically acceptable salt thereof.

The preparation of COMPOUND I and the use thereof, especially as an anti-tumor agent, are described in Example 21 of European patent application EP-A-0 564 409, the contents of which is hereby incorporated by reference, and in corresponding applications and patents in numerous other countries, e.g. in US patent 5,521,184 and in Japanese patent 2706682.

Pharmaceutically acceptable salts of COMPOUND I are pharmaceutically acceptable acid addition salts, like for example with inorganic acids, such as hydrochloric acid, sulfuric acid or a phosphoric acid, or with suitable organic carboxylic or sulfonic acids, for example aliphatic mono- or di-carboxylic acids, such as trifluoroacetic acid, acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, fumaric acid, hydroxymaleic acid, malic acid, tartaric acid, citric acid or oxalic acid, or amino acids such as arginine or lysine, aromatic carboxylic acids, such as benzoic acid, 2-phenoxy-benzoic acid, 2-acetoxy-benzoic acid, salicylic acid, 4-aminosalicylic acid, aromatic-aliphatic carboxylic acids, such as mandelic acid or cinnamic acid, heteroaromatic carboxylic acids, such as nicotinic acid or isonicotinic acid, aliphatic

sulfonic acids, such as methane-, ethane- or 2-hydroxyethane-sulfonic acid, or aromatic sulfonic acids, for example benzene-, p-toluene- or naphthalene-2-sulfonic acid.

The monomethanesulfonic acid addition salt of COMPOUND I (hereinafter "COMPOUND I mesylate" or "imatinib mesylate" or "COMPOUND I monomethanesulfonate") and a preferred crystal form thereof, e.g. the β-crystal form, are described in PCT patent application WO99/03854 published on January 28, 1999.

Possible pharmaceutical preparations, containing an effective amount of COMPOUND I or a pharmaceutically acceptable salt thereof are also described in WO99/03854 , the contents of which is incorporated herein by reference.

According to formula I, the following suitable, preferred, more preferred or most preferred aspects of the invention may be incorporated independently, collectively or in any combination.

Preference is also given to pyrimidylaminobenzamides of formula I, wherein py is 3-pyridyl and wherein the radicals mutually independently of each other have the following meanings:

- R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, acyloxy-lower alkyl, carboxy-lower alkyl, lower alkoxycarbonyl-lower alkyl, or phenyl-lower alkyl; more preferably hydrogen;
- R_2 represents hydrogen, lower alkyl, optionally substituted by one or more identical or different radicals R_3, cycloalkyl, benzcycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted;
- R_3 represents hydroxy, lower alkoxy, acyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amino, mono- or disubstituted amino, cycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted; and
- R_4 represents lower alkyl, especially methyl.

A preferred pyrimidylaminobenzamide of formula I is 4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(4-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl] benzamide, also known as "nilotinib".

The general terms used hereinbefore and hereinafter preferably have within the context of this disclosure the following meanings, unless otherwise indicated:

The prefix "lower" denotes a radical having up to and including a maximum of 7, especially up to and including a maximum of 4 carbon atoms, the radicals in question being either linear or branched with single or multiple branching.

Where the plural form is used for compounds, salts, and the like, this is taken to mean also a single compound, salt, or the like.

Lower alkyl is preferably alkyl with from and including 1 up to and including 7, preferably from and including 1 to and including 4, and is linear or branched; preferably, lower alkyl is butyl, such as n-butyl, sec-butyl, isobutyl, tert-butyl, propyl, such as n-propyl or isopropyl, ethyl or methyl. Preferably lower alkyl is methyl, propyl or tert-butyl.

Lower acyl is preferably formyl or lower alkylcarbonyl, in particular acetyl.

An aryl group is an aromatic radical which is bound to the molecule via a bond located at an aromatic ring carbon atom of the radical. In a preferred embodiment, aryl is an aromatic radical having 6 to 14 carbon atoms, especially phenyl, naphthyl, tetrahydronaphthyl, fluorenyl or phenanthrenyl, and is unsubstituted or substituted by one or more, preferably up to three, especially one or two substituents, especially selected from amino, mono- or disubstituted amino, halogen, lower alkyl, substituted lower alkyl, lower alkenyl, lower alkynyl, phenyl, hydroxy, etherified or esterified hydroxy, nitro, cyano, carboxy, esterified carboxy, alkanoyl, benzoyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amidino, guanidino, ureido, mercapto, sulfo, lower alkylthio, phenylthio, phenyl-lower alkylthio, lower alkylphenylthio, lower alkylsulfinyl, phenylsulfinyl, phenyl-lower alkylsulfinyl, lower alkylphenylsulfinyl, lower alkylsulfonyl, phenylsulfonyl, phenyl-lower alkylsulfonyl, lower alkylphenylsulfonyl, halogen-lower alkylmercapto, halogen-lower alkylsulfonyl, such as

especially trifluoromethanesulfonyl, dihydroxybora (-$B(OH)_2$), heterocyclyl, a mono- or bicyclic heteroaryl group and lower alkylene dioxy bound at adjacent C-atoms of the ring, such as methylene dioxy. Aryl is more preferably phenyl, naphthyl or tetrahydronaphthyl, which in each case is either unsubstituted or independently substituted by one or two substituents selected from the group comprising halogen, especially fluorine, chlorine, or bromine; hydroxy; hydroxy etherified by lower alkyl, e.g. by methyl, by halogen-lower alkyl, e.g. trifluoromethyl, or by phenyl; lower alkylene dioxy bound to two adjacent C-atoms, e.g. methylenedioxy, lower alkyl, e.g. methyl or propyl; halogen-lower alkyl, e.g. trifluoromethyl; hydroxy-lower alkyl, e.g. hydroxymethyl or 2-hydroxy-2-propyl; lower alkoxy-lower alkyl; e.g. methoxymethyl or 2-methoxyethyl; lower alkoxycarbonyl-lower alkyl, e.g. methoxy-carbonylmethyl; lower alkynyl, such as 1-propynyl; esterified carboxy, especially lower alkoxycarbonyl, e.g. methoxycarbonyl, n-propoxy carbonyl or iso-propoxy carbonyl; N-mono-substituted carbamoyl, in particular carbamoyl monosubstituted by lower alkyl, e.g. methyl, n-propyl or iso-propyl; amino; lower alkylamino, e.g. methylamino; di-lower alkylamino, e.g. dimethylamino or diethylamino; lower alkylene-amino, e.g. pyrrolidino or piperidino; lower oxaalkylene-amino, e.g. morpholino, lower azaalkylene-amino, e.g. piperazino, acylamino, e.g. acetylamino or benzoylamino; lower alkylsulfonyl, e.g. methylsulfonyl; sulfamoyl; or phenylsulfonyl.

A cycloalkyl group is preferably cyclopropyl, cyclopentyl, cyclohexyl or cycloheptyl, and may be unsubstituted or substituted by one or more, especially one or two, substituents selected from the group defined above as substituents for aryl, most preferably by lower alkyl, such as methyl, lower alkoxy, such as methoxy or ethoxy, or hydroxy, and further by oxo or fused to a benzo ring, such as in benzcyclopentyl or benzcyclohexyl.

Substituted alkyl is alkyl as last defined, especially lower alkyl, preferably methyl; where one or more, especially up to three, substituents may be present, primarily from the group selected from halogen, especially fluorine, amino, N-lower alkylamino, N,N-di-lower alkylamino, N-lower alkanoylamino, hydroxy, cyano, carboxy, lower alkoxycarbonyl, and phenyl-lower alkoxycarbonyl. Trifluoromethyl is especially preferred.

Mono- or disubstituted amino is especially amino substituted by one or two radicals selected independently of one another from lower alkyl, such as methyl; hydroxy-lower alkyl, such as 2-hydroxyethyl; lower alkoxy lower alkyl, such as methoxy ethyl; phenyl-lower alkyl, such as

benzyl or 2-phenylethyl; lower alkanoyl, such as acetyl; benzoyl; substituted benzoyl, wherein the phenyl radical is especially substituted by one or more, preferably one or two, substituents selected from nitro, amino, halogen, N-lower alkylamino, N,N-di-lower alkylamino, hydroxy, cyano, carboxy, lower alkoxycarbonyl, lower alkanoyl, and carbamoyl; and phenyl-lower alkoxycarbonyl, wherein the phenyl radical is unsubstituted or especially substituted by one or more, preferably one or two, substituents selected from nitro, amino, halogen, N-lower alkylamino, N,N-di-lower alkylamino, hydroxy, cyano, carboxy, lower alkoxycarbonyl, lower alkanoyl, and carbamoyl; and is preferably N-lower alkylamino, such as N-methylamino, hydroxy-lower alkylamino, such as 2-hydroxyethylamino or 2-hydroxypropyl, lower alkoxy lower alkyl, such as methoxy ethyl, phenyl-lower alkylamino, such as benzylamino, N,N-di-lower alkylamino, N-phenyl-lower alkyl-N-lower alkylamino, N,N-di-lower alkylphenylamino, lower alkanoylamino, such as acetylamino, or a substituent selected from the group comprising benzoylamino and phenyl-lower alkoxycarbonylamino, wherein the phenyl radical in each case is unsubstituted or especially substituted by nitro or amino, or also by halogen, amino, N-lower alkylamino, N,N-di-lower alkylamino, hydroxy, cyano, carboxy, lower alkoxycarbonyl, lower alkanoyl, carbamoyl or aminocarbonylamino. Disubstituted amino is also lower alkylene-amino, e.g. pyrrolidino, 2-oxopyrrolidino or piperidino; lower oxaalkylene-amino, e.g. morpholino, or lower azaalkylene-amino, e.g. piperazino or N-substituted piperazino, such as N-methylpiperazino or N-methoxycarbonylpiperazino.

Halogen is especially fluorine, chlorine, bromine, or iodine, especially fluorine, chlorine, or bromine.

Etherified hydroxy is especially C_8-C_{20}alkyloxy, such as n-decyloxy, lower alkoxy (preferred), such as methoxy, ethoxy, isopropyloxy, or tert-butyloxy, phenyl-lower alkoxy, such as benzyloxy, phenyloxy, halogen-lower alkoxy, such as trifluoromethoxy, 2,2,2-trifluoroethoxy or 1,1,2,2-tetrafluoroethoxy, or lower alkoxy which is substituted by mono- or bicyclic hetero-aryl comprising one or two nitrogen atoms, preferably lower alkoxy which is substituted by imidazolyl, such as 1H-imidazol-1-yl, pyrrolyl, benzimidazolyl, such as 1-benzimidazolyl, pyridyl, especially 2-, 3- or 4-pyridyl, pyrimidinyl, especially 2-pyrimidinyl, pyrazinyl, isoquinolinyl, especially 3-isoquinolinyl, quinolinyl, indolyl or thiazolyl.

Esterified hydroxy is especially lower alkanoyloxy, benzoyloxy, lower alkoxycarbonyloxy, such as tert-butoxycarbonyloxy, or phenyl-lower alkoxycarbonyloxy, such as benzyloxycarbonyloxy.

Esterified carboxy is especially lower alkoxycarbonyl, such as tert-butoxycarbonyl, iso-propoxycarbonyl, methoxycarbonyl or ethoxycarbonyl, phenyl-lower alkoxycarbonyl, or phenyloxycarbonyl.

Alkanoyl is primarily alkylcarbonyl, especially lower alkanoyl, e.g. acetyl.

N-Mono- or N,N-disubstituted carbamoyl is especially substituted by one or two substituents independently selected from lower alkyl, phenyl-lower alkyl and hydroxy-lower alkyl, or lower alkylene, oxa-lower alkylene or aza-lower alkylene optionally substituted at the terminal nitrogen atom.

A mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted, refers to a heterocyclic moiety that is unsaturated in the ring binding the heteroaryl radical to the rest of the molecule in formula I and is preferably a ring, where in the binding ring, but optionally also in any annealed ring, at least one carbon atom is replaced by a heteroatom selected from the group consisting of nitrogen, oxygen and sulfur; where the binding ring preferably has 5 to 12, more preferably 5 or 6 ring atoms; and which may be unsubstituted or substituted by one or more, especially one or two, substituents selected from the group defined above as substituents for aryl, most preferably by lower alkyl, such as methyl, lower alkoxy, such as methoxy or ethoxy, or hydroxy. Preferably the mono- or bicyclic heteroaryl group is selected from 2H-pyrrolyl, pyrrolyl, imidazolyl, benzimidazolyl, pyrazolyl, indazolyl, purinyl, pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, 4H-quinolizinyl, isoquinolyl, quinolyl, phthalazinyl, naphthyridinyl, quinoxalyl, quinazolinyl, quinnolinyl, pteridinyl, indolizinyl, 3H-indolyl, indolyl, isoindolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, triazolyl, tetrazolyl, furazanyl, benzo[d]pyrazolyl, thienyl and furanyl. More preferably the mono- or bicyclic heteroaryl group is selected from the group consisting of pyrrolyl, imidazolyl, such as 1H-imidazol-1-yl, benzimidazolyl, such as 1-benzimidazolyl, indazolyl, especially 5-indazolyl, pyridyl, especially 2-, 3- or 4-pyridyl, pyrimidinyl, especially 2-pyrimidinyl, pyrazinyl, isoquinolinyl, especially 3-isoquinolinyl,

quinolinyl, especially 4- or 8-quinolinyl, indolyl, especially 3-indolyl, thiazolyl, benzo[d]pyrazolyl, thienyl, and furanyl. In one preferred embodiment of the invention the pyridyl radical is substituted by hydroxy in ortho position to the nitrogen atom and hence exists at least partially in the form of the corresponding tautomer which is pyridin-(1H)2-one. In another preferred embodiment, the pyrimidinyl radical is substituted by hydroxy both in position 2 and 4 and hence exists in several tautomeric forms, e.g. as pyrimidine-(1H, 3H)2,4-dione.

Heterocyclyl is especially a five, six or seven-membered heterocyclic system with one or two heteroatoms selected from the group comprising nitrogen, oxygen, and sulfur, which may be unsaturated or wholly or partly saturated, and is unsubstituted or substituted especially by lower alkyl, such as methyl, phenyl-lower alkyl, such as benzyl, oxo, or heteroaryl, such as 2-piperazinyl; heterocyclyl is especially 2- or 3-pyrrolidinyl, 2-oxo-5-pyrrolidinyl, piperidinyl, N-benzyl-4-piperidinyl, N-lower alkyl-4-piperidinyl, N-lower alkyl-piperazinyl, morpholinyl, e.g. 2- or 3-morpholinyl, 2-oxo-1H-azepin-3-yl, 2-tetrahydrofuranyl, or 2-methyl-1,3-dioxolan-2-yl.

Pyrimidylaminobenzamides within the scope of formula I, wherein Py is 3-pyridyl and the process for their manufacture are disclosed in WO 04/005281, the contents of which is incorporated herein by reference.

Pharmaceutically acceptable salts of pyrimidylaminobenzamides of formula I, wherein Py is 3-pyridyl, are especially those disclosed in WO2007/015871. In one preferred embodiment nilotinib is employed in the form of its hydrochloride monohydrate. WO2007/015870 discloses certain polymorphs of nilotinib and pharmaceutically acceptable salts thereof useful for the present invention.

The pyrimidylaminobenzamides of formula I, wherein Py is 3-pyridyl, can be administered by any route including orally, parenterally, e.g., intraperitoneally, intravenously, intramuscularly, subcutaneously, intratumorally, or rectally, or enterally. Preferably, the pyrimidyl-aminobenzamides of formula I, wherein py is 3-pyridyl, is administered orally, preferably at a daily dosage of 50-2000 mg. A preferred oral daily dosage of nilotinib is 200 - 1200 mg, e.g. 800 mg, administered as a single dose or divided into multiple doses, such as twice daily dosing.

The term "treatment" as used herein means curative treatment and prophylactic treatment.

The term "curative" as used herein means efficacy in treating ongoing episodes of pulmonary hypertension, especially pulmonary arterial hypertension,.

The term "prophylactic" means the prevention of the onset or recurrence of pulmonary hypertension, especially pulmonary arterial hypertension,.

Throughout this specification and in the claims that follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The invention also pertains to a pharmaceutical preparation for the treatment of pulmonary arterial hypertension comprising COMPOUND I.

Short Description of the Figures

Fig. 1 depicts the change in pulmonary vascular resistance (PVR) in patients obtaining Imatinib mesylate.

Fig. 2 depicts the change in pulmonary vascular resistance (PVR) in patients obtaining placebo.

Fig. 3 depicts the change in cardiac output (CO) in patients obtaining Imatinib mesylate.

Fig. 4 depicts the change in cardiac output (CO) in patients obtaining placebo.

Fig. 5 depicts the change in pulmonary artery pressure (PAP) in patients obtaining Imatinib mesylate.

Fig. 6 depicts the change in pulmonary artery pressure (PAP) in patients obtaining placebo.

Fig. 7 depicts the patient disposition of the intention to treat (ITT) population.

Fig. 8 depicts the mean change from baseline in pulmonary hemodynamics after 6 months of treatment with imatinib or placebo. (a) mean pulmonary artery pressure (PAPm); (b) cardiac output (CO); (c) pulmonary vascular resistance (PVR); (d) 6-minute walking distance (6MWD).

Fig. 9 depicts the mean change from baseline to study end in pulmonary hemodynamics in patients randomized to imatinib or placebo, stratified by baseline PVR ≥1,000 dynes.sec.cm^{-5} (imatinib N=8; placebo N=12) or <1,000 dynes.sec.cm^{5} (imatinib N=12; placebo N=9). (a) mean pulmonary artery pressure (PAPm); (b) cardiac output (CO); (c) pulmonary vascular resistance (PVR); (d) 6-minute walking distance (6MWD).

World Health Organization Classification of Functional Status of Patients With Pulmonary Hypertension

The status of their pulmonary hypertension can be assessed in patients according to the World Health Organization (WHO) classification (modified after the New York Association Functional Classification) as detailed below:

Class I – Patients with pulmonary hypertension but without resulting limitation of physical activity. Ordinary physical activity does not cause undue dyspnea or fatigue, chest pain or near syncope.

Class II – Patients with pulmonary hypertension resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity causes undue dispend or fatigue, chest pain or near syncope.

Class III – Patients with pulmonary hypertension resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary activity causes undue dyspnea or fatigue, chest pain or near syncope.

Class IV – Patients with pulmonary hypertension with inability to carry out any physical activity without symptoms. These patients manifest signs of right heart failure. Dyspnea and/or fatigue may even be present at rest. Discomfort is increased by any physical activity.

In a preferred embodiment of the present invention the medicament is designated for treating pulmonary arterial hypertension in patients who failed prior therapy, especially after receiving at least one prostanoid, endothelin antagonist or PDE V inhibitor.

In a further preferred embodiment of the present invention the medicament is designated for treating pulmonary arterial hypertension in patients who are more severely affected, in particular in patients with Class II to Class IV functional status, more preferably Class III or IV functional status.

In a further preferred embodiment of the present invention the medicament is designated for treating pulmonary arterial hypertension in patients who are harboring BMPR2 mutations.

In a more general aspect, the present invention provides a method of treating humans suffering from

(a) idiopathic or primary pulmonary hypertension,
(b) familial hypertension,
(c) pulmonary hypertension secondary to, but not limited to, connective tissue disease, congenital heart defects (shunts), pulmonary fibrosis, portal hypertension, HIV infection, sickle cell disease, drugs and toxins (e.g., anorexigens, cocaine), chronic hypoxia, chronic pulmonary obstructive disease, sleep apnea, and schistosomiasis,
(d) pulmonary hypertension associated with significant venous or capillary involvement (pulmonary veno-occlusive disease, pulmonary capillary hemangiomatosis),
(e) secondary pulmonary hypertension that is out of proportion to the degree of left ventricular dysfunction,
(f) persistent pulmonary hypertension in newborn babies,

especially in patients who failed prior PAH therapy, which comprises administering to said human in need of such treatment a dose effective against the respective disorder of 4-methylpiperazin-1-ylmethyl)-N-[4-methyl-3-(4-pyridin-3-yl)pyrimidin-2-ylamino)phenyl]-benzamide or a pyrimidylaminobenzamide of formula I or a pharmaceutically acceptable salt thereof, respectively, preferably a dose effective against the respective disorder of a pyrimidylaminobenzamide of formula I or a pharmaceutically acceptable salt thereof.

Depending on species, age, individual condition, mode of administration, and the clinical picture in question, effective doses, for example daily doses of about 100-1000 mg,

preferably 200-600 mg, especially 400 mg of COMPOUND I, are administered to warm-blooded animals of about 70 kg bodyweight. For adult patients a starting dose corresponding to 400 mg of COMPOUND I free base daily can be recommended. For patients with an inadequate response after an assessment of response to therapy with a dose corresponding to 400 mg of COMPOUND I free base daily, dose escalation can be safely considered and patients may be treated as long as they benefit from treatment and in the absence of limiting toxicities.

The invention relates also to a method for administering to a human subject having pulmonary arterial hypertension a pharmaceutically effective amount of COMPOUND I or a pyrimidylaminobenzamide of formula I or a pharmaceutically acceptable salt thereof to the human subject. Preferably, COMPOUND I or a pyrimidylaminobenzamide of formula I or a pharmaceutically acceptable salt thereof is administered once daily for a period exceeding 3 months. The invention relates especially to such method wherein a daily dose of COMPOUND I mesylate corresponding to 100 to 1000 mg, e.g. 200 to 800 mg, especially 400-600 mg, preferably 400 mg, of COMPOUND I free base is administered.

According to the present invention, COMPOUND I is preferably in the form of the monomethanesulfonate salt, e.g. in the β-crystal form of the monomethanesulfonate salt.

The invention relates to a method of treating a warm-blooded animal, especially a human, suffering from pulmonary hypertension, especially pulmonary arterial hypertension, comprising administering to the animal a combination which comprises (a) COMPOUND I or a pyrimidylaminobenzamide of formula I and (b) at least one compound selected from compounds indicated for the treatment of pulmonary arterial hypertension, such as calcium channel antagonists, e.g. nifedipine, e.g. 120 to 240 mg/d, or diltiazem, e.g. 540 to 900 mg/d, prostacyclin, the prostacyclin analogues iloprost, flolan and treprostinil, adenosine, inhaled nitric oxide, anticoagulants, e.g. warfarin, digoxin, endothelin receptor blockers, e.g. bosentan, phosphodiesterease inhibitors, e.g. sildenafil, norepinephrine, angiotensin-converting enzyme inhibitors e.g. enalapril or diuretics; a combination comprising (a) and (b) as defined above and optionally at least one pharmaceutically acceptable carrier for simultaneous, separate or sequential use, in particular for the treatment of pulmonary arterial hypertension; a pharmaceutical composition comprising such a combination; the use of such a combination for the preparation of a medicament for the delay of progression or treatment

of pulmonary arterial hypertension; and to a commercial package or product comprising such a combination.

The structure of the active agents identified by code nos., generic or trade names may be taken from the actual edition of the standard compendium "The Merck Index" or from databases, e.g. Patents International (e.g. IMS World Publications). The corresponding content thereof is hereby incorporated by reference.

When the combination partners employed in the combinations as disclosed herein are applied in the form as marketed as single drugs, their dosage and mode of administration can take place in accordance with the information provided on the package insert of the respective marketed drug in order to result in the beneficial effect described herein, if not mentioned herein otherwise.

It can be shown by established test models that the COMPOUND I or a pyrimidylamino-benzamide of formula I or a pharmaceutically acceptable salt thereof, results in a more effective prevention or preferably treatment of pulmonary arterial hypertension. COMPOUND I or a pharmaceutically acceptable salt thereof has significant fewer side effects as a current therapy. Furthermore, COMPOUND I or a pharmaceutically acceptable salt thereof, results in beneficial effects in different aspects, such as, e.g. incremental benefit with time or to reverse the disease process. COMPOUND I, or a pharmaceutically acceptable salt thereof, shows an unexpected high potency to prevent or eliminate pulmonary arterial hypertension, because of its unexpected multifunctional activity, and its activity on different aspects of pulmonary arterial hypertension.

The person skilled in the pertinent art is fully enabled to select a relevant test model to prove the hereinbefore and hereinafter indicated therapeutic indications and beneficial effects (i.e. good therapeutic margin, and other advantages mentioned herein). The pharmacological activity is, for example, demonstrated by *in vitro* and *in vivo* test procedures such as rodent models of pulmonary arterial hypertension, or in a clinical study as essentially described hereinafter. The following Examples illustrate the invention described above, but are not, however, intended to limit the scope of the invention in any way.

Example 1: A randomized, double-blind, placebo-controlled study to evaluate the safety and efficacy of six months treatment with the tyrosine kinase inhibitor Imatinib Mesylate for the treatment of pulmonary arterial hypertension

Primary objectives

• To assess the safety and tolerability of oral Imatinib Mesylate compared with placebo in patients with pulmonary arterial hypertension (PAH).

• To evaluate efficacy of oral Imatinib Mesylate as measured by an improvement in 6-minute walk test.

Secondary objective(s)

• To evaluate the efficacy of oral Imatinib Mesylate as measured by improvement in clinical status (assessment of WHO class and Borg Score), and changes in pulmonary homodynamic parameters (including mean pulmonary arterial pressure, mean Pulmonary Artery Wedge pressure, Systolic Arterial Pressure, Heart Rate, and Cardiac Output, Pulmonary Vascular Resistance, Systemic Vascular Resistance), time to clinical worsening, changes in plasma biomarker levels.

Design:

In the study a total of 60 patients with PAH was enrolled who have been shown to be deteriorating on, or not tolerating, standard therapy (prostanoids (i.v., s.c., inhaled), endothelin-1 antagonists, or PDE-5 inhibitors), but may still be continuing with the standard therapy. Eligible patients were randomized to receive oral Imatinib Mesylate 200mg daily rising to 400mg after 2 weeks, or matching placebo. Treatment continued for 6 months with weekly visits for the first four weeks followed by monthly visits up to six months (Week 24). Safety and efficacy assessments were performed at pre-specified time points up to Week 24. Male or female patients aged 18 years or older with pulmonary arterial hypertension according to the Venice Classification (2003) of either primary (idiopathic), familial or secondary to systemic sclerosis (excluding those with marked pulmonary fibrosis) and a WHO classification of II to IV (maximum of 50% of patients will be class IV) were included. Patients harboring a mutation in BMPR2 gene were identified. Patients had been receiving therapy with prostanoids (i.v., s.c., inhaled), endothelin-1 antagonists, or PDE-5 inhibitors, but have shown to be deteriorating (not improving on), or not tolerating this standard

therapy. PAH medication had been stable for at least 3 months prior to inclusion in the study (Baseline visit). Imatinib Mesylate was applied as 100 mg clinical trial formulation capsules for oral administration and matching placebo capsules. The 200 mg dose consisted of 2 x 100mg capsules or 2 x matching placebo. The 400 mg dose consisted of 4 x 100 mg capsules or matching placebo. Patients were instructed to take the study drug once daily with a meal and a large glass (8oz/200 mL) of water and not to chew the medication, but to swallow it whole.

Efficacy assessments

• Six minute walk test and Borg Score: Screening, Baseline, Week 4, Week 8, Week 12, Week 16, Week 20, Week 24/Study Completion.

• WHO Assessment: Screening, Baseline, Week 4, Week 8, Week 12, Week 16, Week 20, Week 24/Study Completion

• Hemodynamic parameters (PAP, PAWP, SAP, HR, CO, PVR and SVR) from right sided heart catheritization: Baseline and Week 24/Study Completion.

Results

Table 1 - Change in Key Variables Baseline to Study End (mean [percent])

	mPAP (mmHg)	CO (l/min)	PVR (dyne/s · cm)–5	PCWP (mmHg)	6MW
IM N=19	-6.42 (-11%)	0.83 (20%)	-300 (-29%)	-0.4 (-4%)	18.1 (5%)
Placebo N=21	-2.66 (-4%)	0.11 (3%)	-81 (-8%)	1.4 (19%)	-12 (-3%)
IM - Placebo	-3.75 (7%)	0.71 (17%)	218 (-21%)	1.8 (23%)	30 (8%)
P Value	0.27	0.017	0.029	0.07	0.06

Table 2 - Change by Baseline PVR / PVR<1000

	mPAP	PVR	CO	6MW
IM (N=7)	-4.61538	-173.769	0.291538	3.2
PL (N=12)	-3.25	-74.375	0.57375	14.4

Table 3 - Change by Baseline PVR / PVR>1000

	mPAP	PVR	CO	6MW
IM (N=12)	-8.57143	-596.571	1.271429	70
PL(N=9)	-6.33333	-121.75	0.229167	-32

6MW: 6-minute walk test; CO: cardiac output; IM: Imatinib mesylate;; PAP: pulmonary arterial pressure; PCWP: pulmonary capillary wedge pressure; PL: placebo; PVR: pulmonary vascular resistance

The study demonstrates a clear beneficial change in pulmonary vascular resistance (PVR), cardiac output (CO) and six minute walk in response to Imatinib mesylate compared to placebo. A trend in reduction in pulmonary artery pressure (PAP) was also seen. There was a difference in the number of deaths (5 versus 3) in favor of Imatinib mesylate.

Example 2: A randomized, double-blind, placebo-controlled trial to evaluate imatinib treatment for patients with severe pulmonary arterial hypertension with inadequate response to established therapy

Introduction

Pulmonary arterial hypertension (PAH) (defined as a mean pulmonary artery pressure [PAPm] of ≥25 mmHg at rest or 30 mmHg with exercise, mean pulmonary capillary wedge pressure [PCWPm] ≤15 mmHg and pulmonary vascular resistance [PVR] > 240 dynes.sec.cm^{-5}) leads to progressive increases in pulmonary vascular resistance (PVR), right ventricular failure and death if untreated. Estimated 1 and 3 year survival rates in idiopathic PAH (IPAH) without targeted therapy are 68% and 48%, respectively.

Current drug therapy recommendations for PAH vary depending on the patient's functional class (FC, World Health Organization's [WHO] Modification for Pulmonary Hypertension of the New York Heart Association Functional Class). The phosphodiesterase type 5 (PDE5) inhibitor sildenafil, oral endothelin receptor antagonists (ERAs) bosentan, ambrisentan and sitaxsentan, and prostacyclin analogues epoprostenol (intravenous), iloprost (inhaled) and treprostinil (subcutaneous or intravenous) are approved for patients in FC II-IV. Patients in FC III or IV who fail to improve or deteriorate with monotherapy can be treated with combination therapy, atrial septostomy and/or transplantation (lung or heart/lung). However, to date, none of these therapeutic options cure PAH despite improvement in survival; PAH remains a progressive and frequently fatal condition. Two recent meta-analyses highlighted the beneficial effects of prostacyclin analogues, ERAs and PDE5 inhibitors on exercise capacity and some other clinical endpoints in PAH patients, while only the most recent report by Galie et al. provided evidence of improved survival by the aforementioned treatments.

Pathological changes in the pulmonary arteries of patients with PAH include the formation of plexiform lesions, and smooth muscle and fibroblast proliferation leading to vascular obstruction. Platelet-derived growth factor (PDGF) is a vascular smooth muscle cell mitogen activating signal transduction pathways associated with smooth muscle hyperplasia in pulmonary hypertension. PDGF and its receptor (PDGFR) have been implicated in the pathobiology of pulmonary hypertension in animal studies and in patients with PAH thereby offering a potential new target for treatment.

Imatinib, a tyrosine kinase inhibitor that inhibits PDGFR α and β kinases, Abl, DDR and c-KIT, may therefore prove efficacious in the treatment of PAH. Several case reports have provided promising results thus warranting further study of imatinib in PAH.

In the present study the effects of imatinib versus placebo were compared in a randomized, double-blind, placebo-controlled pilot study in PAH patients who had not adequately improved with prostacyclin analogues, ERAs, PDE5 inhibitors and/or combinations of these therapies.

Methods

1. Study objectives and design

The primary objectives were to assess the safety and tolerability of imatinib compared with placebo in PAH patients and to evaluate its efficacy using the 6-minute walk test (6MW test). Secondary objectives included changes in hemodynamic variables, and in FC.

Patients (≥18 years) in FC II-IV with idiopathic or familial PAH, or PAH associated with systemic sclerosis or congenital heart disease (WHO group I) and PVR > 300 dynes.sec.cm^{-5} were eligible. Patients were on stable PAH medication(s) for > 3 months before enrolment. Females of child-bearing potential used double-barrier contraception.

Patients with other causes of PAH were excluded. Patients were not allowed to use nonspecific PDE inhibitors, chronic inhaled nitric oxide therapy or catecholamines during the study. Additional exclusion criteria included: participation in another clinical trial within 3 months, donation or loss of blood (>400 mL) within 8 weeks or a history of another significant illness within 4 weeks. Patients were also excluded if they had pre-existing lung disease, coagulation disorders, thrombocytopenia, major bleeding or intracranial haemorrhage, history of latent bleeding risk, elevated liver transaminases (>4 times upper limit of normal [ULN]), elevated bilirubin (>2 times ULN), elevated serum creatinine (>200 µmol/L), history of elevated intracranial pressure, pregnancy, breast feeding, sickle cell anaemia, history of clinically significant drug allergy or atopic allergy, history of immunodeficiency, hepatitis B or C, or history of drug or alcohol abuse. Patients were excluded if they had known hypersensitivity to the study drug, any condition that could alter the study drug pharmacokinetics or put them at risk, if their underlying disease was likely to

result in failure to survive the study, or if they were unable to perform the 6MW test due to a condition other than PAH. Eligible patients were enrolled at 7 centres in Germany, the United Kingdom, Austria, and the United States and randomized 1:1 to treatment with either imatinib or placebo.

The study was designed, implemented and reported in accordance with International Conference on Harmonization (ICH) Harmonized Tripartite Guidelines for Good Clinical Practice and all applicable local regulations (including European Directive 2001/83/EC and US Code of Federal Regulations Title 21) and with the ethical principles laid down in the Declaration of Helsinki. This study was approved by institutional review boards at all centres and all patients signed informed consent before enrolment. All deaths and safety data were reviewed throughout the study by an external data safety monitoring board.

2. Interventions

Treatment with imatinib (or placebo) was initiated at a dose of 200 mg orally once daily for the first two weeks of treatment. If treatment was well tolerated, the dose was increased to 400 mg/day. If the 400 mg dose was not well tolerated, down-titration to 200 mg was permitted. Patients and investigators were blind to the treatment allocation. The blinding could be broken in an emergency.

3. Efficacy assessments

The primary efficacy outcome was the between-group difference in the 6MW distance (6MWD) at baseline and at 6 months. Complete hemodynamic parameters were assessed with standard techniques. FC was classified according to the WHO modification of the NYHA criteria for pulmonary hypertension.

4. Exploratory Analysis

To generate new hypotheses and to identify patient subgroups that may respond better than other subgroups to imatinib, additional subgroup analyses were conducted in patients with PVR values of $\geq$1,000 vs. <1,000 dynes.sec.cm^{-5} (the median of the data).

5. Safety assessments

Monitoring of blood cell counts, hepatic and renal function parameters, echocardiography and cardiac magnetic resonance imaging (in selected centres) was conducted during the study. Patients were also interviewed via regular telephone calls between scheduled study visits.

6. Statistical analysis

The planned sample size of 60 subjects was selected to address both safety and the primary efficacy outcome (6MWD). For the primary efficacy outcome it was estimated that the study had 80% power to detect a 55 m increase in the 6MWD with 95% confidence (two-sided $p<0.05$), based on a standard deviation (SD) of 75 m.

Analyses were conducted within the intention-to-treat (ITT) population, which consisted of all patients who received at least one dose of study medication. Dropouts were excluded from the analysis The primary efficacy analysis (6MWD) was performed using analysis of covariance (ANCOVA) with baseline value as a covariate. ANCOVAs were also used to assess between-group differences in pulmonary hemodynamics and blood gases. Missing data were not imputed so only subjects with assessment both at baseline and post-treatment were included in the ANCOVA analysis. FC was compared using Fisher's test.

In addition, exploratory analyses (post-hoc) were performed in subgroups classified according to baseline PVR values $\geq$or < 1,000 dynes.sec.cm^{-5} at baseline (i.e. the median PVR in the study).

Results

1. Disposition and baseline characteristics:

Fifty-nine patients (40 female; 19 male) were enrolled with 42 (71.2%) completing the 6 month study (Figure 7). The majority of dropouts not related to death were to worsening of PAH. Baseline characteristics were similar between the two treatment groups (Table 4). Overall, patients had a mean age of 44.3 years, mean weight of 68.7 kg and mean body mass index of 24.6 kg/m^2. Fifty five of the 59 patients were Caucasian and 78% had idiopathic PAH (Table 4). At baseline, 79% of the imatinib- and 81% of the placebo-group patients were receiving combination therapy (Table 4).

Table 4. Baseline characteristics of the intention to treat (ITT) population

	Imatinib (N=28)	**Placebo** (N=31)
Age (years), mean (SD)	44.4 (15.3)	44.2 (15.7)
Gender, male/female, n (%)	10 (36)/ 8 (64)	9 (29)/22 (71)
Ethnicity, n (%)		
Caucasian	26 (92)	29 (94)
Asian	0	1 (3)
Black	1 (4)	0
Pacific Islander	0	1 (3)
Hispanic	1 (4)	0
Weight (kg), mean (SD)	70.1 (14.7)	67.4 (23.4)
Height (cm), mean (SD)	168.6 (8.8)	164.3 (8.6)
Diagnosis, n (%)		
Idiopathic pulmonary hypertension	21 (75)	25 (81)
Familial pulmonary hypertension	2 (7)	0
Pulmonary hypertension secondary to systemic sclerosis	1 (4)	5 (16)
Other	4 (14)	1 (3)
WHO classification, n (%)*		
Class II	13 (48)	7 (23)
Class III	12 (44)	23 (74)
Class IV	2 (7)	1 (3)
PAH specific treatments, n (%)		
ERA alone	2 (7)	4 (13)
Sildenafil alone	2 (7)	0 (0)
Prostacylin analog alone	2 (7)	1 (3)
ERA + prostacylin analog	1 (4)	3 (10)
ERA + sildenafil	12 (43)	9 (29)
Sildenafil + prostacyclin analog	5 (18)	3 (10)
ERA + sildenafil + prostacyclin	4 (14)	10 (32)
Calcium channel blocker	0	1 (3)

SD: standard deviation; PH: pulmonary hypertension; prostacyclin analogues (iloprost, epoprostenol, trepostinil and beraprost); ERA: endothelin receptor antagonists (bosentan and ambrisentan)
*WHO assessment was not available for one patient receiving imatinib

2. Efficacy outcomes:

The mean (±SD) 6MWD did not significantly change in the imatinib group vs. placebo (+22±63 vs. –1.0±53 m; mean treatment difference 21.7 m ; 95% CI (-13.0, 56.5); p=0.21) (Table 5; Figure 8). There was, however, a significant decrease in PVR (mean treatment difference -230.7 dynes ; 95% CI (-383.7, -77.8; p=0.004) and increase in cardiac output (CO; mean treatment difference 0.68 L/min ; 95% CI (0.10, 1.26; p=0.02) in imatinib recipients compared with placebo (Figure 8). There was no significant difference in PAPm (Figure 8) or change in FC between imatinib and placebo treated patients (data not shown).

There was an increase in arterial and mixed venous oxygen saturation ($p<0.05$) with imatinib. Systemic arterial oxygen saturation increased from 88±9% to 93±5% with imatinib treatment compared with no change with placebo (92±4% at baseline vs. 92±3% at end of study) (mean treatment difference 2.4%; 95% CI (0.5, 4.3)); mixed venous oxygen saturation increased from 58±10% to 65±7% with imatinib treatment (consistent with the increase in CO) compared with a decrease with placebo (61±6% at baseline vs. 57±9% at end of study) (mean treatment difference 7.0%; 95% CI (2.1, 11.9)).

Table 5. Six-minute walking distance (6MWD) observed at baseline and end of study, and changes from baseline following imatinib and placebo therapy in patients with PAH. The change is expressed as the average alteration in 6MWD from baseline.

	Imatinib		Placebo		Treatment difference (m) [b]	p-value [b]
	Distance walked (m), mean (SD)	Change vs. baseline (m) [a] mean (SD)	Distance walked (m), mean (SD)	Change vs. baseline (m) [a] mean (SD)		
Baseline	392 (89) N=28	—	369 (118) N=29	—	—	—
Study end	419 (85) N=21	22 (63) N=21	399 (86) N=22	–1 (53) N=21	21.7	0.21

[a] Patients with both a baseline and end of study assessment.

[b] ANCOVA of ITT population

3. Exploratory subgroup analyses:

In patients with a baseline PVR ≥1,000 dynes.sec.cm^{-5}, there was a substantial improvement between baseline and study end for PAPm, CO, PVR and 6MWD in the imatinib group compared with placebo (Figure 9). However, among patients with a baseline PVR < 1,000 dynes.sec.cm^{-5}, no major differences between baseline and study end for PAPm, CO, PVR or 6MWD were observed (Figure 9).

4. Safety and tolerability:

The most common adverse events (AEs) observed in this clinical study were as expected for this population and this drug. The most common AEs reported in the imatinib group were nausea (N=14; 50%), headache (N=10; 35.7%) and peripheral edema (N=7; 25.0%). These

AEs did not lead to discontinuation of study drug. Nausea was controlled by taking the medication with food. A total of 21 (75%) patients in the imatinib group and 24 (77%) patients in the placebo group reported AEs of mild intensity, 20 (71%) in the imatinib group and 19 (61%) in the placebo group patients reported AEs of moderate intensity, and 9 (32%) patients in the imatinib group and 5 (16%) patients in the placebo group reported AEs of severe intensity. Serious AEs (SAEs) were reported for 11 imatinib recipients (39%) and 7 placebo recipients (23%). SAEs in the imatinib group included cardiac arrest (N=2), vertigo (n=1), pancreatitis (N=1), catheter related complication (N=1), liver dysfunction (N=2), dizziness (N=1), presyncope (N=1), syncope (N=1), haemoptysis (N=1), worsening pulmonary hypertension (N=3), and arterial rupture (N=1). SAEs in the placebo group included atrial flutter (N=1), cardiac arrest (N=2), right ventricular failure (N=2), general physical health deterioration (N=1), fluid retention (N=1), dizziness (N=1), and worsening pulmonary hypertension (N=3).

Overall there was a fall in the haemoglobin levels with imatinib (151±14 to 128±16 g/L, SD) and a rise in hemoglobin levels with placebo (143±25 to 152±25 g/L). There were no relevant changes over time on the following variables: white blood cell count, platelet count albumin, alkaline phosphatase, total bilirubin, calcium, cholesterol, creatinine, g-GT, glucose, lactate dehydrogenase, inorganic phosphorus, lipase, amylase, potassium, total protein, C-reactive protein, glutamate oxalacetate transaminase, glutamate pyruvate transaminase, sodium, triglycerides, urea, and uric acid.

There were three deaths in each group. Two additional patients died in the placebo group within 2 months of completing the study. One patient in the imatinib group and one patient in the placebo group had rupture of the pulmonary artery (fatal in both cases).

Discussion

This is the first randomized, double-blind, placebo controlled trial to assess the safety, tolerability and efficacy of the tyrosine kinase inhibitor imatinib in patients with PAH. Although imatinib appeared safe and well tolerated over a 6 month period, the primary efficacy parameter (6MWD) did not improve in patients randomized to imatinib compared with placebo, despite significant improvement in secondary endpoints.

Treatment efficacy

Overall, 59 patients were enrolled. As per study protocol, only patients on background treatment with at least one PAH specific drug (i.e. prostacyclin analogues, ERAs, PDE5 inhibitors) who had not adequately improved were enrolled (56% of patients were receiving two drugs and 24% receiving three drugs at baseline). This may have contributed to the reduced improvement in 6MWD observed in this study compared with previous studies in which only treatment naïve patients were included. In clinical trials in which background specific medications have been allowed, the overall improvement in 6MWD has been less than in the treatment naïve trials.

Safety aspects

It has been suggested that inhibition of the ABL tyrosine kinase pathway may infrequently induce myocardial damage in patients receiving long-term treatment with imatinib for chronic myelogenous leukemia (CML). However, a long-term, multicenter study in a large population of patients with CML showed an acceptable safety profile for imatinib. A review of all patients receiving imatinib shows that 0.5% of patients per year developed incident congestive cardiac failure (no risk factors present). In patients with CML receiving imatinib, 0.4% of patients per year develop congestive cardiac failure compared with 0.75% per year for patients receiving interferon gamma plus Ara-C. Considering the potential for cardiotoxicity which could be even more problematic for patients with PAH, regular assessments of cardiac function by echocardiography and measurements of serum cardiac troponin levels were performed in this trial. Overall, there were no signals indicating a potential detrimental effect of imatinib on myocardial function when compared to the overall safety profile of the placebo group. In contrast, some of the beneficial effect of imatinib on PVR reduction appeared to be due to improvements in CO, suggestive of improved right ventricular contractility in patients with PAH. Nonetheless, cardiac safety remains a key concern with other kinase inhibitors, such as sunitinib.

Exploratory subgroup analysis

Although no significant increases in 6MWD were observed with imatinib compared with placebo, significant improvements in CO and PVR were observed. These observations led us to undertake a post-hoc analysis stratifying patients by baseline PVR. In patients with baseline PVR $\geq$1,000 dynes.sec.cm^{-5}, there was a substantial improvement from baseline to study end for 6MWD, PVR, and CO in the imatinib group, when compared with placebo (Figure 9). This was not observed in the patients with PVR levels <1,000 dynes.sec.cm^{-5}.

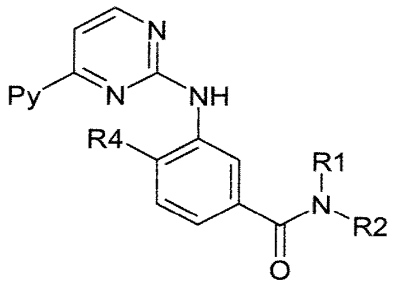
However, these results have to be interpreted with caution as this was an unplanned analysis. In addition, tyrosine kinase inhibitors are not recognized to have any significant vasodilator or inotropic effects, with their effects considered anti-proliferative and pro-apoptotic. One hypothesis that could explain the current study results is that for treatment with imatinib to be effective, a certain degree of disease severity (i.e. vascular proliferation) may be needed. However, as these data are hypothesis generating, it cannot be excluded that less severe patients with PAH may also benefit from long-term imatinib therapy via a preventive mechanism.

Conclusion and perspective

The results of this pilot study suggest that imatinib is safe and well tolerated in patients with PAH. In addition, the efficacy analyses provide proof of concept supporting the use of agents targeting proliferative growth factor pathways in PAH.

Claims:

1. Use of 4-(4-methylpiperazin-1-ylmethyl)-N-[4-methyl-3-(4-pyridin-3-yl)pyrimidin-2-ylamino)phenyl]-benzamide or a pharmaceutically acceptable salt thereof or a pyrimidylaminobenzamide of formula I



(I)

wherein

Py denotes 3-pyridyl,

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, acyloxy-lower alkyl, carboxy-lower alkyl, lower alkoxycarbonyl-lower alkyl, or phenyl-lower alkyl;

R_2 represents hydrogen, lower alkyl, optionally substituted by one or more identical or different radicals R_3, cycloalkyl, benzcycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted; and

R_3 represents hydroxy, lower alkoxy, acyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amino, mono- or disubstituted amino, cycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted;

or wherein R_1 and R_2 together represent alkylene with four, five or six carbon atoms optionally mono- or disubstituted by lower alkyl, cycloalkyl, heterocyclyl, phenyl, hydroxy, lower alkoxy, amino, mono- or disubstituted amino, oxo, pyridyl, pyrazinyl or pyrimidinyl; benzalkylene with four or five carbon atoms; oxaalkylene with one oxygen and three or four carbon atoms; or azaalkylene with one nitrogen and three or four carbon atoms wherein nitrogen is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, lower

alkoxycarbonyl-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, N-mono- or N,N-disubstituted carbamoyl-lower alkyl, cycloalkyl, lower alkoxycarbonyl, carboxy, phenyl, substituted phenyl, pyridinyl, pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen, lower alkyl, or halogen;

or a pharmaceutically acceptable salt thereof, for the manufacture of a medicament for treating pulmonary arterial hypertension (PAH) in patients who failed prior PAH therapy.

2. The use according to claim 1, wherein 4-(4-methylpiperazin-1-ylmethyl)-N-[4-methyl-3-(4-pyridin-3-yl)pyrimidin-2-ylamino)phenyl]-benzamide or a pharmaceutically acceptable salt thereof is used.

3. The use according to claim 2 wherein 4-(4-methylpiperazin-1-ylmethyl)-N-[4-methyl-3-(4-pyridin-3-yl)pyrimidin-2-ylamino)phenyl]-benzamide is used in the form of the mono-methanesulfonate salt.

4. The use according to claim 1, wherein a pyrimidylaminobenzamide of formula I, wherein the radicals and symbols have the meaning as defined in claim 1 or a pharmaceutically acceptable salt thereof, is used.

5. The use according to claim 4, wherein the pyrimidylaminobenzamide is 4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(4-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl] benzamide.

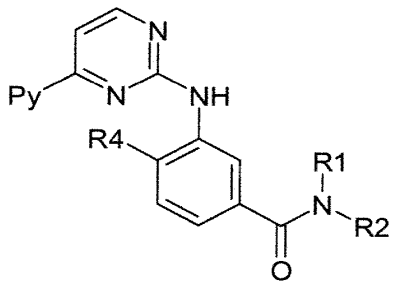
6. The use according to claim 5, wherein 4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(4-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl] benzamide is used in the form of its hydrochloride monohydrate.

7. The use according to any one of claims 1 to 6, wherein prior PAH therapy included receiving at least one prostanoid, endothelin antagonist or PDE V inhibitor.

8. The use according to any one of claims 1 to 6, wherein the medicament is designated for treating PAH in patients who are more severely affected.

9. The use according to any one of claims 1 to 6, wherein the medicament is designated for treating PAH in patients who are harboring BMPR2 mutations.

10. A method of treating humans suffering from pulmonary arterial hypertension (PAH) in patients who failed prior PAH therapy, which comprises administering to a said human in need of such treatment a dose effective against PAH of 4-methylpiperazin-1-ylmethyl)-N-[4-methyl-3-(4-pyridin-3-yl)pyrimidin-2-ylamino)phenyl]-benzamide or a pharmaceutically acceptable salt thereof or a pyrimidylaminobenzamide of formula I



(I)

wherein

Py denotes 3-pyridyl,

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, acyloxy-lower alkyl, carboxy-lower alkyl, lower alkoxycarbonyl-lower alkyl, or phenyl-lower alkyl;

R_2 represents hydrogen, lower alkyl, optionally substituted by one or more identical or different radicals R_3, cycloalkyl, benzcycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted; and

R_3 represents hydroxy, lower alkoxy, acyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amino, mono- or disubstituted amino, cycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted;

or wherein R_1 and R_2 together represent alkylene with four, five or six carbon atoms optionally mono- or disubstituted by lower alkyl, cycloalkyl, heterocyclyl, phenyl, hydroxy, lower alkoxy, amino, mono- or disubstituted amino, oxo, pyridyl, pyrazinyl or pyrimidinyl;

benzalkylene with four or five carbon atoms; oxaalkylene with one oxygen and three or four carbon atoms; or azaalkylene with one nitrogen and three or four carbon atoms wherein nitrogen is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, lower alkoxycarbonyl-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, N-mono- or N,N-disubstituted carbamoyl-lower alkyl, cycloalkyl, lower alkoxycarbonyl, carboxy, phenyl, substituted phenyl, pyridinyl, pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen, lower alkyl, or halogen;

or a pharmaceutically acceptable salt thereof.

11. A method of treating humans suffering from

(a) idiopathic or primary pulmonary hypertension,

(b) familial hypertension,

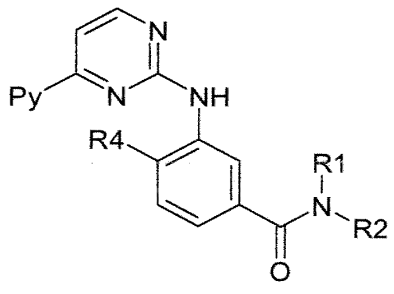
(c) pulmonary hypertension secondary to, but not limited to, connective tissue disease, congenital heart defects (shunts), pulmonary fibrosis, portal hypertension, HIV infection, sickle cell disease, drugs and toxins (e.g., anorexigens, cocaine), chronic hypoxia, chronic pulmonary obstructive disease, sleep apnea, and schistosomiasis,

(d) pulmonary hypertension associated with significant venous or capillary involvement (pulmonary veno-occlusive disease, pulmonary capillary hemangiomatosis),

(e) secondary pulmonary hypertension that is out of proportion to the degree of left ventricular dysfunction,

(f) persistent pulmonary hypertension in newborn babies,

which comprises administering to said human in need of such treatment a dose effective against the respective disorder a pyrimidylaminobenzamide of formula I



(I)

wherein

Py denotes 3-pyridyl,

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, acyloxy-lower alkyl, carboxy-lower alkyl, lower alkoxycarbonyl-lower alkyl, or phenyl-lower alkyl;

R_2 represents hydrogen, lower alkyl, optionally substituted by one or more identical or different radicals R_3, cycloalkyl, benzcycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted; and

R_3 represents hydroxy, lower alkoxy, acyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amino, mono- or disubstituted amino, cycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted;

or wherein R_1 and R_2 together represent alkylene with four, five or six carbon atoms optionally mono- or disubstituted by lower alkyl, cycloalkyl, heterocyclyl, phenyl, hydroxy, lower alkoxy, amino, mono- or disubstituted amino, oxo, pyridyl, pyrazinyl or pyrimidinyl; benzalkylene with four or five carbon atoms; oxaalkylene with one oxygen and three or four carbon atoms; or azaalkylene with one nitrogen and three or four carbon atoms wherein nitrogen is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, lower alkoxycarbonyl-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, N-mono- or N,N-disubstituted carbamoyl-lower alkyl, cycloalkyl, lower alkoxycarbonyl, carboxy, phenyl, substituted phenyl, pyridinyl, pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen, lower alkyl, or halogen;

or a pharmaceutically acceptable salt thereof.

Fig. 1

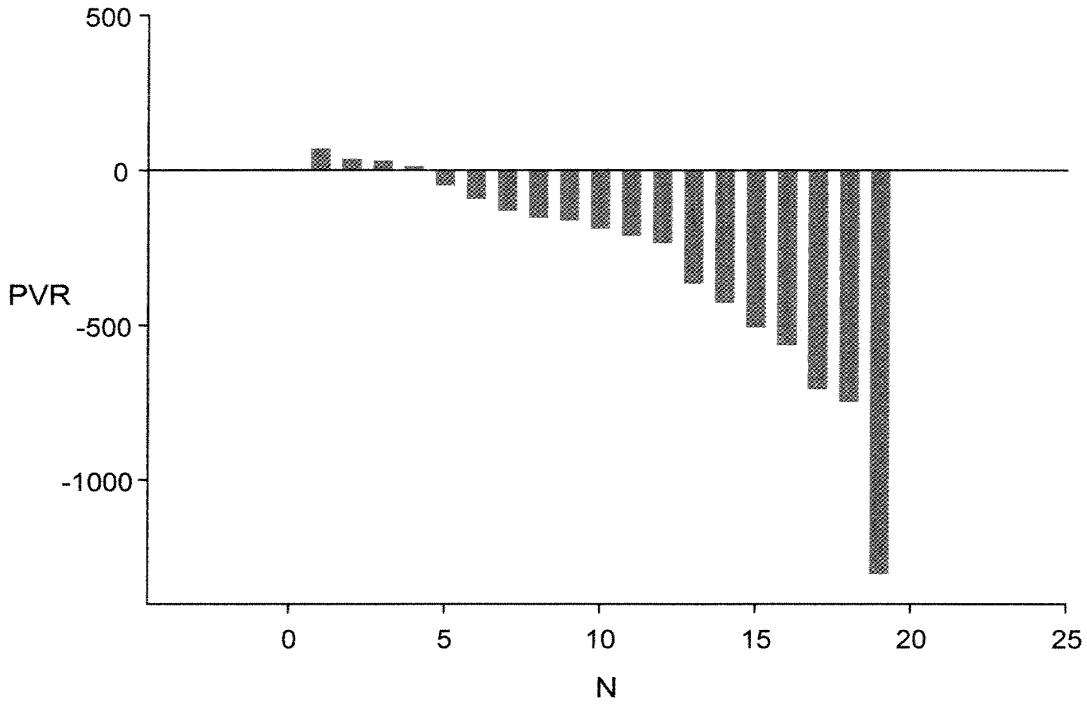


Fig. 2

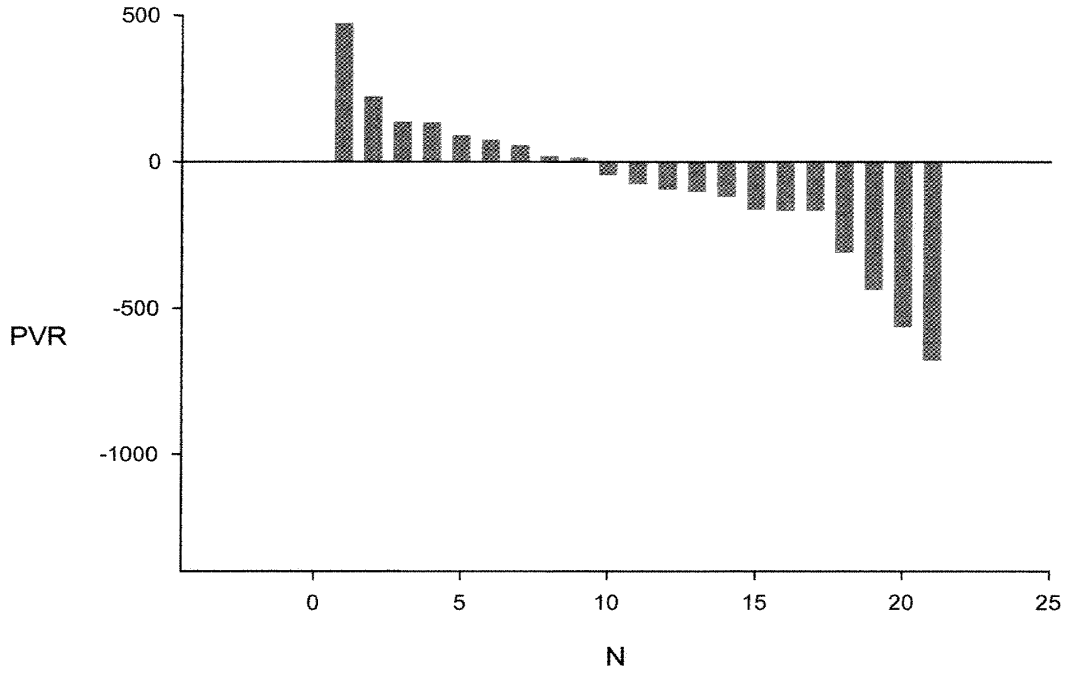


Fig. 3

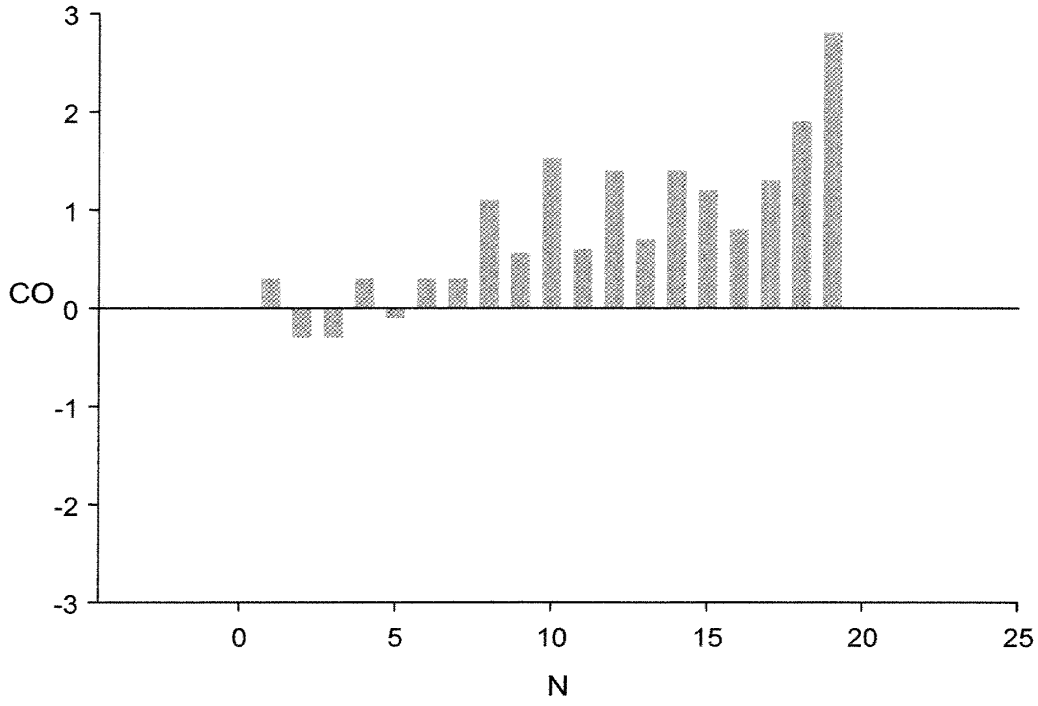


Fig. 4

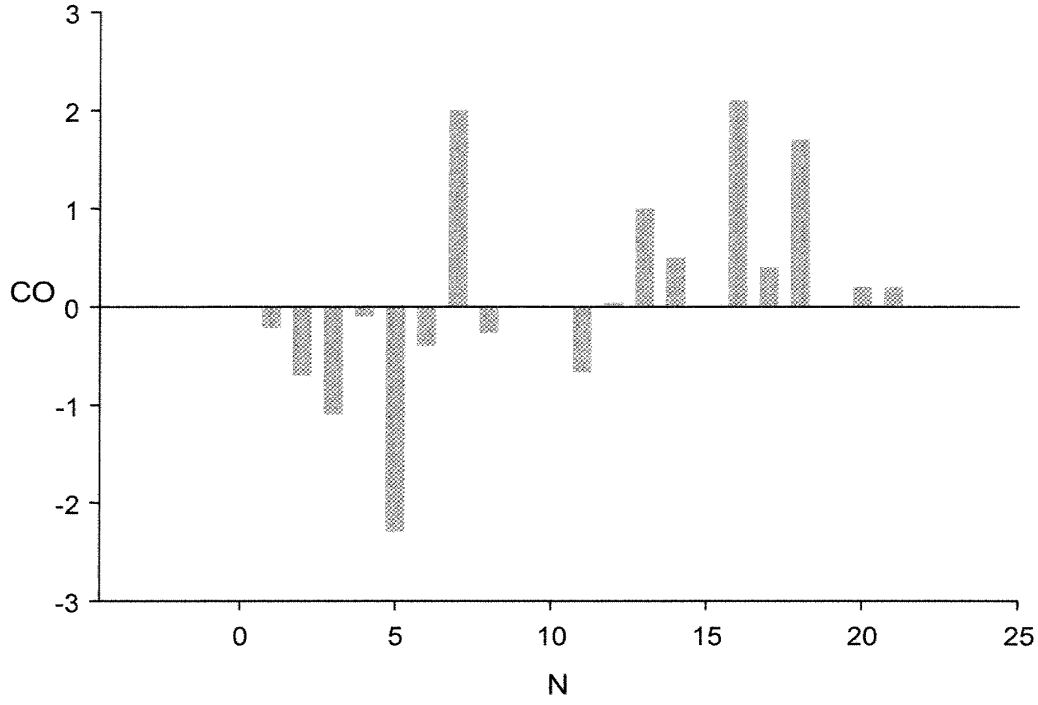
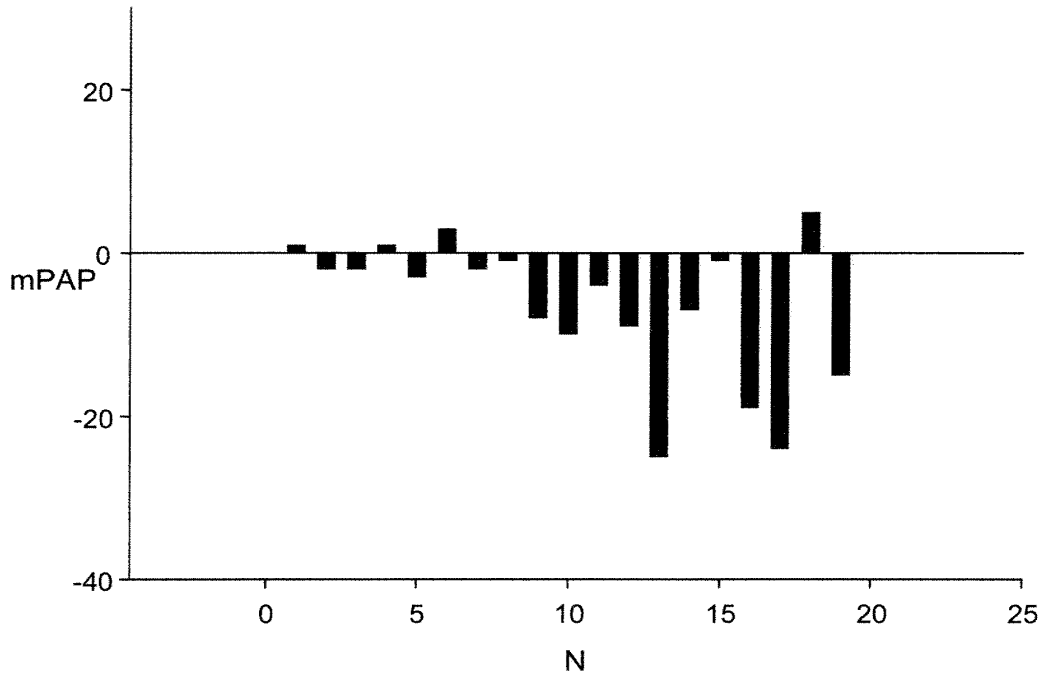


Fig. 5



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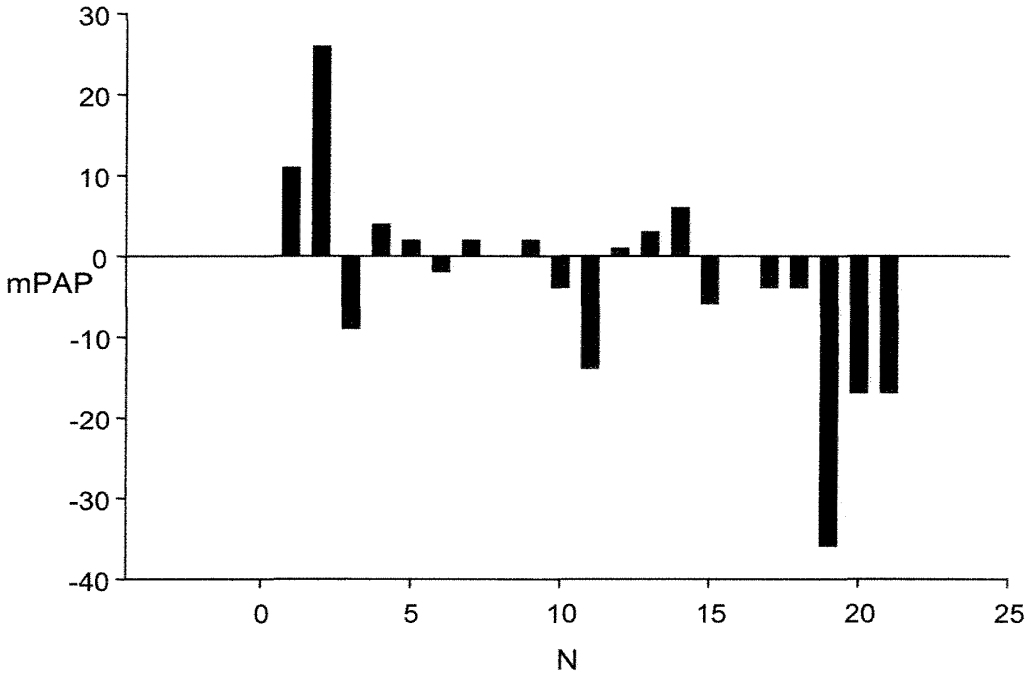

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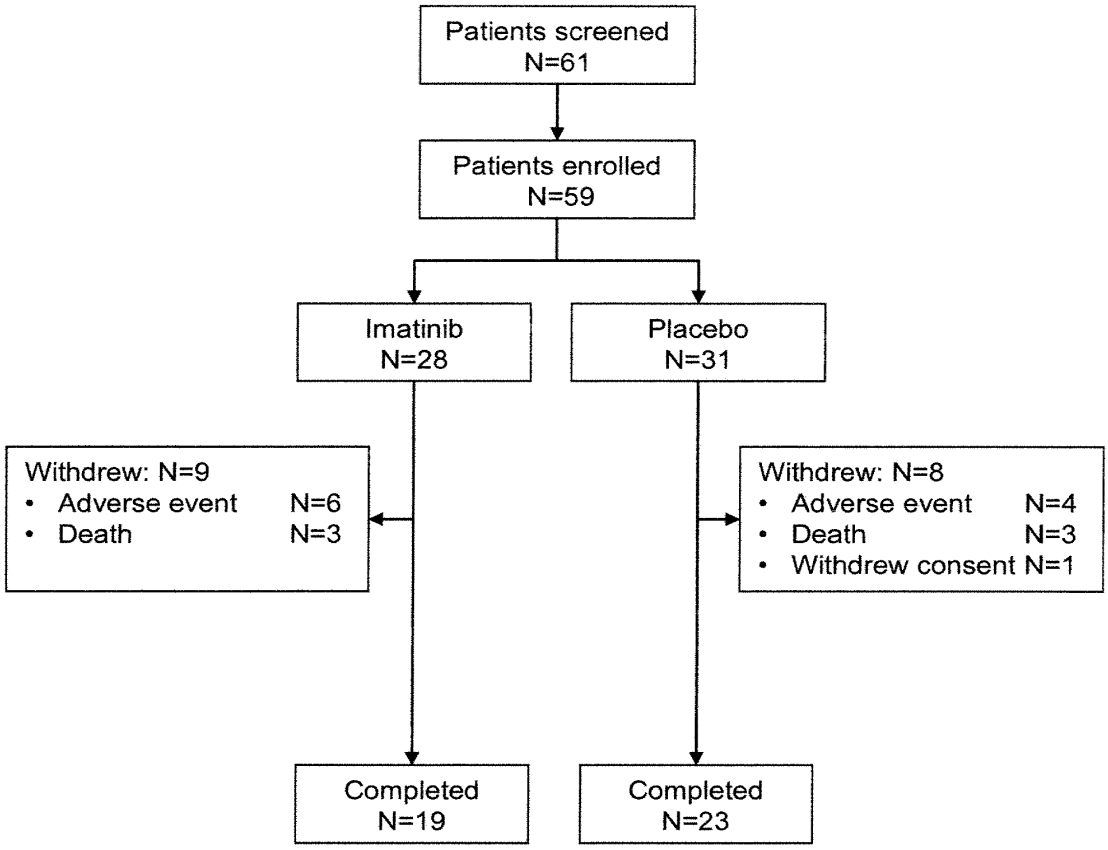
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N=28
Placebo
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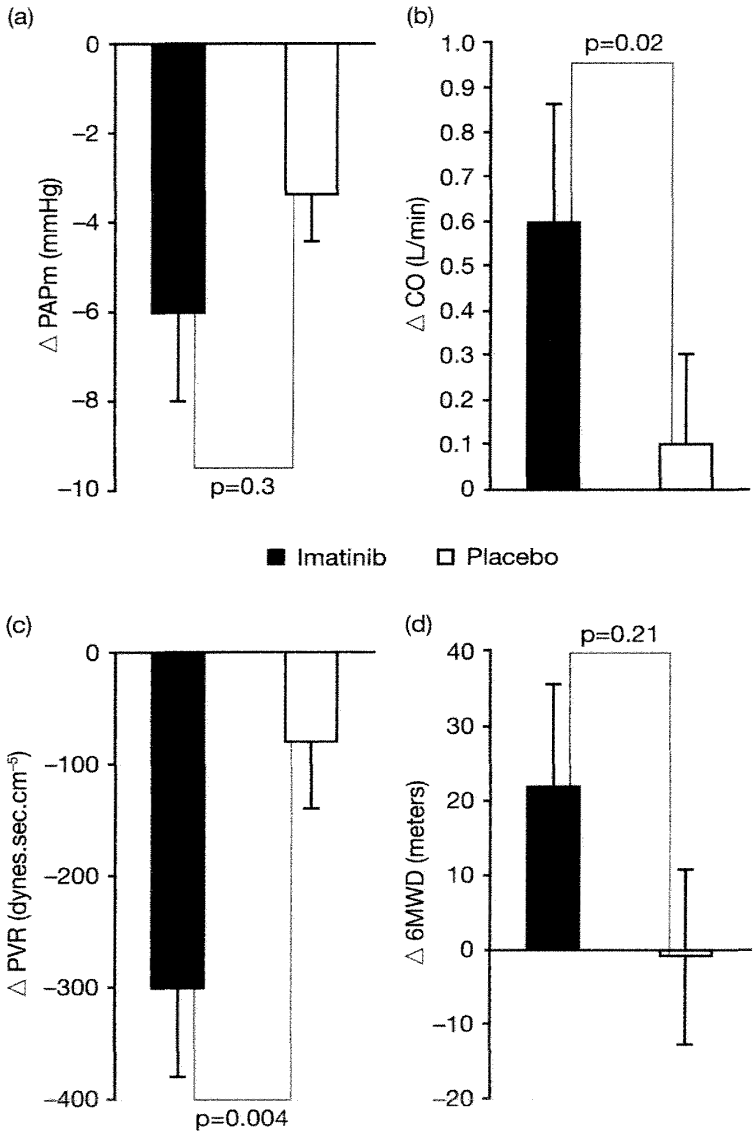
Fig. 8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(b)
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0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
△ CO (L/min)
p=0.02
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Placebo
(c)
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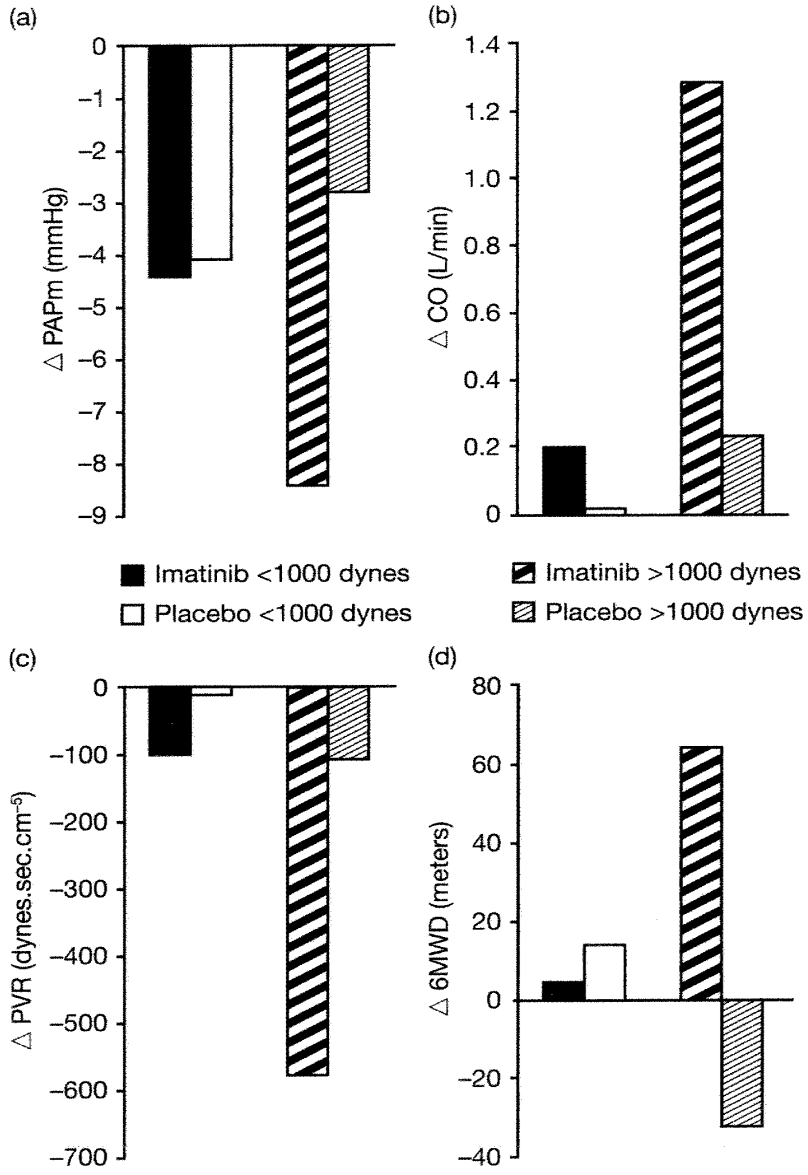
Fig. 9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1.2
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0.8
0.6
0.4
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Δ CO (L/min)
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(d)
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Δ 6MWD (meters)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/053358

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/506 A61P9/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BEILSTEIN Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GHOFRANI, H. A. ET AL.: "Imatinib for the Treatment of Pulmonary Arterial Hypertension" THE NEW ENGLAND JOURNAL OF MEDICINE, vol. 353, no. 13, 2005, pages 1412-1413, XP002550196 the whole document ----- -/--	1-3,7-11

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 October 2009	27/10/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Fax: (+31–70) 340–3016	Sahagún Krause, H

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/053358

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHERMULY R T ET AL: "REVERSAL OF EXPERIMENTAL PULMONARY HYPERTENSION BY PDGF INHIBITION" JOURNAL OF CLINICAL INVESTIGATION, AMERICAN SOCIETY FOR CLINICAL INVESTIGATION, US, vol. 115, no. 10, 1 October 2005 (2005-10-01), pages 2811-2821, XP008056354 ISSN: 0021-9738 abstract	1-3,7,11
Y	WO 2006/079539 A (NOVARTIS AG [CH]; NOVARTIS PHARMA GMBH [AT]; MANLEY PAUL W [CH]; MARTI) 3 August 2006 (2006-08-03) claim 8	1-11
A	ZIMMERMANN J ET AL: "Potent and selective inhibitors of the Abl-kinase: phenylamino-pyrimidine (PAP) derivatives" BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, PERGAMON, ELSEVIER SCIENCE, GB, vol. 7, no. 2, 21 January 1997 (1997-01-21), pages 187-192, XP004135990 ISSN: 0960-894X compound 13	1-11
Y	PERROS, F ET AL.: "Platelet-derived Growth Factor Expression and Function in Idiopathic Pulmonary Arterial Hypertension" AMERICAN JOURNAL OF RESPIRATORY AND CRITICAL CARE MEDICINE, vol. 178, 2008, pages 81-88, XP002550197 the whole document	1-11
Y	WO 2004/005281 A (NOVARTIS AG [CH]; NOVARTIS PHARMA GMBH [AT]; BREITENSTEIN WERNER [CH];) 15 January 2004 (2004-01-15) cited in the application claim 1, page 2, lines 1-2, table in pages 69-70	1-11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2009/053358

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006079539 A	03-08-2006	AU 2006208638 A1	03-08-2006
		BR PI0606872 A2	21-07-2009
		CA 2594687 A1	03-08-2006
		EP 1843771 A2	17-10-2007
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		EP 1532138 A1	25-05-2005
		EP 2100891 A1	16-09-2009
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		US 2008188451 A1	07-08-2008
		US 2006167015 A1	27-07-2006
		US 2007093506 A1	26-04-2007

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	METHODS OF TREATING TERM AND NEAR-TERM NEONATES HAVING HYPOXIC RESPIRATORY FAILURE ASSOCIATED WITH CLINICAL OR ECHOCARDIOGRAPHIC EVIDENCE OF PULMONARY HYPERTENSION
First Named Inventor/Applicant Name:	James S. Baldassarre
Filer:	Daniel Leo Hayes/Anna Goforth
Attorney Docket Number:	I001-0002USC1

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Utility filing Fee (Electronic filing)	4011	1	82	82
Utility Search Fee	2111	1	270	270
Utility Examination Fee	2311	1	110	110
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**462**

Electronic Acknowledgement Receipt

EFS ID:	7869307
Application Number:	12820866
International Application Number:	
Confirmation Number:	2913
Title of Invention:	METHODS OF TREATING TERM AND NEAR-TERM NEONATES HAVING HYPOXIC RESPIRATORY FAILURE ASSOCIATED WITH CLINICAL OR ECHOCARDIOGRAPHIC EVIDENCE OF PULMONARY HYPERTENSION
First Named Inventor/Applicant Name:	James S. Baldassarre
Customer Number:	49584
Filer:	Daniel Leo Hayes/Anna Goforth
Filer Authorized By:	Daniel Leo Hayes
Attorney Docket Number:	I001-0002USC1
Receipt Date:	22-JUN-2010
Filing Date:	
Time Stamp:	17:30:57
Application Type:	Utility under 35 USC 111(a)

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RAM confirmation Number	4610
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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

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	Document Description	**Start**	**End**
	Specification	1	22
	Claims	23	26
	Abstract	27	27

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3	Petition for review by the Office of Petitions.	MV7373.PDF	666553 0c7f42055fa30bb30cfd8c5459bc8d9c6474037d	no	18

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5	Oath or Declaration filed	MV9702.PDF	242838 c9dab52f718c9656218126c5aa04f7dbef3a7a15	no	3

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6	Miscellaneous Incoming Letter	MV7393.PDF	56200 7bbfdec693e2c90f91d16bd31833262833d4d5d5	no	1

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7	Information Disclosure Statement (IDS) Filed (SB/08)	MV7391.PDF	605641 cb401cf02bcdaa4321bc627ff6e8c7fa14e3ac9a	no	10

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

METHODS OF TREATING TERM AND NEAR-TERM NEONATES HAVING HYPOXIC RESPIRATORY FAILURE ASSOCIATED WITH CLINICAL OR ECHOCARDIOGRAPHIC EVIDENCE OF PULMONARY HYPERTENSION

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Patent Application No. 12/494,598, entitled "Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension", filed on June 30, 2009, incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] INOmax®, (nitric oxide) for inhalation is an approved drug product for the treatment of term and near-term (>34 weeks gestation) neonates having hypoxic respiratory failure associated with clinical or echocardiographic evidence of pulmonary hypertension.

[0003] The use of inhaled NO (iNO) has been studied and reported in the literature. (Kieler-Jensen M et al., 1994, Inhaled Nitric Oxide in the Evaluation of Heart Transplant Candidates with Elevated Pulmonary Vascular Resistance, *J Heart Lung Transplantation* 13:366-375; Pearl RG et al., 1983, Acute Hemodynamic Effects of Nitroglycerin in Pulmonary Hypertension, *American College of Physicians* 99:9-13; Ajami GH et al., 2007, Comparison of the Effectiveness of Oral Sildenafil Versus Oxygen Administration as a Test for Feasibility of Operation for Patients with Secondary Pulmonary Arterial Hypertension, *Pediatr Cardiol*; Schulze-Neick I et al., 2003, Intravenous Sildenafil Is a Potent Pulmonary Vasodilator in Children With Congenital Heart Disease, *Circulation* 108(Suppl II):II-167-II-173; Lepore JJ et al., 2002, Effect of Sildenafil on the Acute Pulmonary Vasodilator Response to Inhaled Nitric Oxide in Adults with Primary Pulmonary Hypertension, *The American Journal of Cardiology* 90:677-680; and Ziegler JW et al., 1998, Effects of Dipyridamole and Inhaled Nitric Oxide in Pediatric Patients with Pulmonary Hypertension, *American Journal of Respiratory and Critical Care Medicine* 158:1388-95).

SUMMARY OF THE INVENTION

[0004] One aspect of the invention relates to a pre-screening methodology or protocol having exclusionary criteria to be evaluated by a medical provider prior to treatment of a patient with iNO. One objective of the invention is to evaluate and possibly exclude from treatment patients eligible for treatment with iNO, who have pre-existing left ventricular dysfunction (LVD). Patients who have pre-existing LVD may experience, and are at risk of, an increased rate of adverse events or serious adverse events (e.g., pulmonary edema) when treated with iNO. Such patients may be characterized as having a pulmonary capillary wedge pressure (PCWP) greater than 20 mm Hg, and should be evaluated on a case-by-case basis with respect to the benefit versus risk of using iNO as a treatment option.

[0005] Accordingly, one aspect of the invention includes a method of reducing the risk or preventing the occurrence, in a human patient, of an adverse event (AE) or a serious adverse event (SAE) associated with a medical treatment comprising inhalation of nitric oxide, said method comprising the steps or acts of (a) providing pharmaceutically acceptable nitric oxide gas to a medical provider; and, (b) informing the medical provider that excluding human patients who have pre-existing left ventricular dysfunction from said treatment reduces the risk or prevents the occurrence of the adverse event or the serious adverse event associated with said medical treatment.

[0006] Further provided herein is a method of reducing the risk or preventing the occurrence, in a human patient, of an adverse event or a serious adverse event associated with a medical treatment comprising inhalation of nitric oxide, said method comprising the steps or acts of (a.) providing pharmaceutically acceptable nitric oxide gas to a medical provider; and, (b.) informing the medical provider that human patients having pre-existing left ventricular dysfunction experience an increased risk of serious adverse events associated with said medical treatment.

[0007] Another aspect of the invention is a method of reducing one or more of an AE or a SAE in an intended patient population in need of being treated with iNO comprising the steps or acts of (a.) identifying a patient eligible for iNO treatment; (b) evaluating and screening the patient to identify if the patient has pre-existing LVD, and (c) excluding from iNO treatment a patient identified as having pre-existing LVD.

[0008] Another aspect of the invention is a method of reducing the risk or preventing the occurrence, in a patient, of one or more of an AE or a SAE associated with a medical treatment comprising iNO, the method comprising the steps or acts of (a.) identifying a patient in need of receiving iNO treatment; (b.) evaluating and screening the patient to identify if the patient has pre-existing LVD; and (c.)administering iNO if the patient does not have pre-existing LVD, thereby reducing the risk or preventing the occurrence of the AE or the SAE associated with the iNO treatment. Alternatively, step (c) may comprise further evaluating the risk versus benefit of utilizing iNO in a patient where the patients has clinically significant LVD before administering iNO to the patient.

[0009] In an exemplary embodiment of the method, the method further comprises informing the medical provider that there is a risk associated with using inhaled nitric oxides in human patients who have preexisting or clinically significant left ventricular dysfunction and that such risk should be evaluated on a case by case basis.

[0010] In another exemplary embodiment of the method, the method further comprises informing the medical provider that there is a risk associated with using inhaled nitric oxide in human patients who have left ventricular dysfunction.

[0011] In an exemplary embodiment of the methods described herein, a patient having pre-existing LVD is characterized as having PCWP greater than 20 mm Hg.

[0012] In an exemplary embodiment of the method, the patients having pre-existing LVD demonstrate a PCWP $\geq$ 20 mm Hg.

[0013] In another exemplary embodiment of the method, the iNO treatment further comprises inhalation of oxygen (O_2) or concurrent ventilation.

[0014] In another exemplary embodiment of the method, the patients having pre-existing LVD have one or more of diastolic dysfunction, hypertensive cardiomyopathy, systolic dysfunction, ischemic cardiomyopathy, viral cardiomyopathy, idiopathic cardiomyopathy, autoimmune disease related cardiomyopathy, drug-related cardiomyopathy, toxin-related cardiomyopathy, structural heart disease, valvular heart disease, congenital heart disease, or, associations thereof.

[0015] In another exemplary embodiment of the method, the patient population comprises children.

[0016] In another exemplary embodiment of the method, the patient population comprises adults.

[0017] In another exemplary embodiment of the method, the patients who have pre-existing LVD are at risk of experiencing and increased rate of one or more AEs or SAEs selected from pulmonary edema, hypotension, cardiac arrest, electrocardiogram changes, hypoxemia, hypoxia, bradycardia or associations thereof.

[0018] In another exemplary embodiment of the method, the intended patient population in need of being treated with inhalation of nitric oxide has one or more of idiopathic pulmonary arterial hypertension characterized by a mean pulmonary artery pressure (PAPm) > 25 mm Hg at rest, PCWP ≤ 15 mm Hg, and, a pulmonary vascular resistance index (PVRI) > 3 u·m^2; congenital heart disease with pulmonary hypertension repaired and unrepaired characterized by PAPm > 25 mm Hg at rest and PVRI > 3 u·m^2; cardiomyopathy characterized by PAPm > 25 mm Hg at rest and PVRI > 3 u·m^2; or, the patient is scheduled to undergo right heart catheterization to assess pulmonary vasoreactivity by acute pulmonary vasodilatation testing.

[0019] In another exemplary embodiment of any of the above methods, the method further comprises reducing left ventricular afterload to minimize or reduce the risk of the occurrence of an adverse event or serious adverse event being pulmonary edema in the patient. The left ventricular afterload may be minimized or reduced by administering a pharmaceutical dosage form comprising nitroglycerin or calcium channel blocker to the patient. The left ventricular afterload may also be minimized or reduced using an intra-aortic balloon pump.

DETAILED DESCRIPTION OF THE EXEMPLARY EMBODIMENTS

[0020] INOmax® (nitric oxide) for inhalation was approved for sale in the United States by the U.S. Food and Drug Administration ("FDA") in 1999. Nitric oxide, the active substance in INOmax®, is a selective pulmonary vasodilator that increases the partial pressure of arterial oxygen (PaO_2) by dilating pulmonary vessels in better ventilated areas of the lung, redistributing pulmonary blood flow away from the lung regions with low ventilation/perfusion (V/Q) ratios toward regions with normal ratios. INOmax® significantly improves oxygenation, reduces the need for extracorporeal oxygenation and is indicated to be used in conjunction with ventilatory support and other appropriate agents. The current FDA-approved prescribing information for INOmax® is incorporated herein by reference in its entirety.

[0021] INOmax® is a gaseous blend of NO and nitrogen (0.08% and 99.92% respectively for 800 ppm; and 0.01% and 99.99% respectively for 100 ppm) and is supplied in aluminium cylinders as a compressed gas under high pressure. In general, INOmax® is administered to a patient in conjunction with ventilatory support and O_2. Delivery devices suitable for the safe and effective delivery of gaseous NO for inhalation include the INOvent®, INOmax DS®, INOpulse®, INOblender®, or other suitable drug delivery and regulation devices or components incorporated therein, or other related processes, which are described in various patent documents including USPNs 5,558,083; 5,732,693; 5,752,504; 5,732,694; 6,089,229; 6,109,260; 6,125,846; 6,164,276; 6,581,592; 5,918,596; 5,839,433; 7,114,510; 5,417;950; 5,670,125; 5,670,127; 5,692,495; 5,514,204; 7,523,752; 5,699,790; 5,885,621; US Patent Application Serial Nos. 11/355,670 (US 2007/0190184); 10/520,270 (US 2006/0093681); 11/401,722 (US 2007/0202083); 10/053,535 (US 2002/0155166); 10/367,277 (US 2003/0219496); 10/439,632 (US 2004/0052866); 10/371,666 (US 2003/0219497); 10/413,817 (US 2004/0005367); 12/050,826 (US 2008/0167609); and PCT/US2009/045266, all of which are incorporated herein by reference in their entirety.

[0022] Such devices deliver INOmax® into the inspiratory limb of the patient breathing circuit in a way that provides a constant concentration of NO to the patient throughout the inspired breath. Importantly, suitable delivery devices provide continuous integrated monitoring of inspired O_2, NO_2 and NO, a comprehensive alarm system, a suitable power source for uninterrupted NO delivery and a backup NO delivery capability.

[0023] As used herein, the term "children" (and variations thereof) includes those being around 4 weeks to 18 years of age.

[0024] As used herein, the term "adult" (and variations thereof) includes those being over 18 years of age.

[0025] As used herein, the terms "adverse event" or "AE" (and variations thereof) mean any untoward occurrence in a subject, or clinical investigation subject administered a pharmaceutical product (such as nitric oxide) and which does not necessarily have a causal relationship with such treatment. An adverse event can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporarily associated with the use of a medicinal/investigational product, whether or not related to the

investigational product. A relationship to the investigational product is not necessarily proven or implied. However, abnormal values are not reported as adverse events unless considered clinically significant by the investigator.

[0026] As used herein, the terms "adverse drug reaction" or "ADR" (and variations thereof) mean any noxious and unintended response to a medicinal product related to any dose.

[0027] As used herein, the terms "serious adverse event" or "SAE" (or "serious adverse drug reaction" or "serious ADR") (and variations thereof) mean a significant hazard or side effect, regardless of the investigator's opinion on the relationship to the investigational product. A serious adverse event or reaction is any untoward medical occurrence that at any dose: results in death; is life-threatening (which refers to an event/reaction where the patient was at risk of death at the time of the event/reaction, however this does not refer to an event/reaction that hypothetically may have caused death if it were more severe); requires inpatient hospitalization or results in prolongation of existing hospitalization; results in persistent or significant disability/incapacity; is a congenital anomaly/birth defect; or, is a medically important event or reaction. Medical and scientific judgment is exercised in deciding whether reporting is appropriate in other situations, such as important medical events that may not be immediately life threatening or result in death or hospitalization but may jeopardize the subject or may require medical or surgical intervention to prevent one of the other outcomes listed above--these are also considered serious. Examples of such medical events include cancer, allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in hospitalizations, or the development of drug dependency or drug abuse. Serious clinical laboratory abnormalities directly associated with relevant clinical signs or symptoms are also reported.

[0028] Left Ventricular Dysfunction. Patients having pre-existing LVD may be described in general as those with elevated pulmonary capillary wedge pressure, including those with diastolic dysfunction (including hypertensive cardiomyopathy), those with systolic dysfunction, including those with cardiomyopathies (including ischemic or viral cardiomyopathy, or idiopathic cardiomyopathy, or autoimmune disease related cardiomyopathy, and side effects due to drug related or toxic-related cardiomyopathy), or structural heart disease, valvular heart disease, congenital heart disease, idiopathic pulmonary arterial hypertension,

pulmonary hypertension and cardiomyopathy, or associations thereof. Identifying patients with pre-existing LVD is known to those skilled in the medicinal arts, and such techniques for example may include assessment of clinical signs and symptoms of heart failure, or echocardiography diagnostic screening.

[0029] Pulmonary Capillary Wedge Pressure. Pulmonary capillary wedge pressure, or "PCWP", provides an estimate of left atrial pressure. Identifying patients with pre-existing PCWP is known to those skilled in the medicinal arts, and such techniques for example may include measure by inserting balloon-tipped, multi-lumen catheter (also known as a Swan-Ganz catheter). Measure of PCWP may be used as a means to diagnose the severity of LVD (sometimes also referred to as left ventricular failure). PCWP is also a desired measure when evaluating pulmonary hypertension. Pulmonary hypertension is often caused by an increase in pulmonary vascular resistance (PVR), but may also arise from increases in pulmonary venous pressure and pulmonary blood volume secondary to left ventricular failure or mitral or aortic valve disease.

[0030] In cardiac physiology, afterload is used to mean the tension produced by a chamber of the heart in order to contract. If the chamber is not mentioned, it is usually assumed to be the left ventricle. However, the strict definition of the term relates to the properties of a single cardiac myocyte. It is therefore only of direct relevance in the laboratory; in the clinic, the term end-systolic pressure is usually more appropriate, although not equivalent.

[0031] The terms "left ventricular afterload" (and variations thereof) refer to the pressure that the chamber of the heart has to generate in order to eject blood out of the chamber. Thus, it is a consequence of the aortic pressure since the pressure in the ventricle must be greater than the systemic pressure in order to open the aortic valve. Everything else held equal, as afterload increases, cardiac output decreases. Disease processes that increase the left ventricular afterload include increased blood pressure and aortic valve disease. Hypertension (Increased blood pressure) increases the left ventricular afterload because the left ventricle has to work harder to eject blood into the aorta. This is because the aortic valve won't open until the pressure generated in the left ventricle is higher than the elevated blood pressure. Aortic stenosis increases the afterload because the left ventricle has to overcome the pressure gradient caused by the stenotic aortic valve in addition to the blood pressure in order to eject blood into the

aorta. For instance, if the blood pressure is 120/80, and the aortic valve stenosis creates a trans-valvular gradient of 30 mmHg, the left ventricle has to generate a pressure of 110 mmHg in order to open the aortic valve and eject blood into the aorta. Aortic insufficiency increases afterload because a percentage of the blood that is ejected forward regurgitates back through the diseased aortic valve. This leads to elevated systolic blood pressure. The diastolic blood pressure would fall, due to regurgitation. This would result in an increase pulse pressure. Mitral regurgitation decreases the afterload. During ventricular systole, the blood can regurgitate through the diseased mitral valve as well as be ejected through the aortic valve. This means that the left ventricle has to work less to eject blood, causing a decreased afterload. Afterload is largely dependent upon aortic pressure.

[0032] An intra-aortic balloon pump (IABP) is a mechanical device that is used to decrease myocardial oxygen demand while at the same time increasing cardiac output. By increasing cardiac output it also increases coronary blood flow and therefore myocardial oxygen delivery. It consists of a cylindrical balloon that sits in the aorta and counterpulsates. That is, it actively deflates in systole increasing forward blood flow by reducing afterload thus, and actively inflates in diastole increasing blood flow to the coronary arteries. These actions have the combined result of decreasing myocardial oxygen demand and increasing myocardial oxygen supply. The balloon is inflated during diastole by a computer controlled mechanism, usually linked to either an ECG or a pressure transducer at the distal tip of the catheter; some IABPs, such as the Datascope System 98XT, allow for asynchronous counterpulsation at a set rate, though this setting is rarely used. The computer controls the flow of helium from a cylinder into and out of the balloon. Helium is used because its low viscosity allows it to travel quickly through the long connecting tubes, and has a lower risk of causing a harmful embolism should the balloon rupture while in use. Intraaortic balloon counterpulsation is used in situations when the heart's own cardiac output is insufficient to meet the oxygenation demands of the body. These situations could include cardiogenic shock, severe septic shock, post cardiac surgery and numerous other situations.

[0033] Patients eligible for treatment with iNO. In general, patients approved for treatment of iNO are term and near-term (>34 weeks gestation) neonates having hypoxic respiratory failure associated with clinical or echocardiographic evidence of pulmonary hypertension, a condition also known as persistent pulmonary hypertension in the newborn

(PPHN). Due to the selective, non-systemic nature of iNO to reduce pulmonary hypertension, physicians skilled in the art further employ INOmax® to treat or prevent pulmonary hypertension and improve blood O_2 levels in a variety of other clinical settings, including in both pediatric and adult patients suffering from acute respiratory distress syndrome (ARDS), pediatric and adult patients undergoing cardiac or transplant surgeries, pediatric and adult patients for testing to diagnose reversible pulmonary hypertension, and in pediatric patients with congenital diaphragmatic hernia. In most, if not all, of these applications, INOmax® acts by preventing or treating reversible pulmonary vasoconstriction, reducing pulmonary arterial pressure and improving pulmonary gas exchange.

[0034] A small proportion of INOmax® sales stem from its use by clinicians in a premature infant population. In these patients, INOmax® is generally utilized by physicians as a rescue therapy primarily to vasodilate the lungs and improve pulmonary gas exchange. Some physicians speculate that INOmax® therapy may promote lung development and/or reduce or prevent the future development of lung disease in a subset of these patients. Although the precise mechanism(s) responsible for the benefits of INOmax® therapy in these patients is not completely understood, it appears that the benefits achieved in at least a majority of these patients are due to the ability of INOmax® to treat or prevent reversible pulmonary vasoconstriction.

[0035] In clinical practice, the use of INOmax® has reduced or eliminated the use of high risk systemic vasodilators for the treatment of PPHN. INOmax®, in contrast to systemic vasodilators, specifically dilates the pulmonary vasculature without dilating systemic blood vessels. Further, iNO preferentially vasodilates vessels of aveoli that are aerated, thus improving V/Q matching. In contrast, systemic vasodilators may increase blood flow to atelectatic (deflated or collapsed) alveoli, thereby increasing V/Q mismatch and worsening arterial oxygenation. (*See* Rubin LJ, Kerr KM, Pulmonary Hypertension, in *Critical Care Medicine: Principles of Diagnosis and Management in the Adult*, *2d Ed.*, Parillo JE, Dellinger RP (eds.), Mosby, Inc. 2001, pp. 900-09 at 906; Kinsella JP, Abman SH, The Role of Inhaled Nitric Oxide in Persistent Pulmonary Hypertension of the Newborn, in *Acute Respiratory Care of the Neonate: A Self-Study Course*, *2d Ed.*, Askin DF (ed.), NICU Ink Book Publishers, 1997, pp. 369-378 at 372-73).

[0036] INOmax® also possesses highly desirable pharmacokinetic properties as a lung-specific vasodilator when compared to other ostensibly "pulmonary-specific vasodilators." For example, the short half-life of INOmax® allows INOmax® to exhibit rapid "on" and "off" responses relative to INOmax® dosing, in contrast to non-gaseous alternatives. In this way, INOmax® can provide physicians with a useful therapeutic tool to easily control the magnitude and duration of the pulmonary vasodilatation desired. Also, the nearly instantaneous inactivation of INOmax® in the blood significantly reduces or prevents vasodilatation of non-pulmonary vessels.

[0037] The pivotal trials leading to the approval of INOmax® were the CINRGI and NINOS study.

[0038] CINRGI study. (See Davidson et al., March 1998, Inhaled Nitric Oxide for the Early Treatment of Persistent Pulmonary Hypertension of the term Newborn; A Randomized, Double-Masked, Placebo-Controlled, Dose-Response, Multicenter Study; *PEDIATRICS* Vol. 101, No. 3, p. 325).

[0039] This study was a double-blind, randomized, placebo-controlled, multicenter trial of 186 term and near-term neonates with pulmonary hypertension and hypoxic respiratory failure. The primary objective of the study was to determine whether INOmax® would reduce the receipt of extracorporeal membrane oxygenation (ECMO) in these patients. Hypoxic respiratory failure was caused by meconium aspiration syndrome (MAS) (35%), idiopathic persistent pulmonary hypertension of the newborn (PPHN) (30%), pneumonia/sepsis (24%), or respiratory distress syndrome (RDS) (8%). Patients with a mean PaO_2 of 54 mm Hg and a mean oxygenation index (OI) of 44 cm H_2O/mm Hg were randomly assigned to receive either 20 ppm INOmax® (n=97) or nitrogen gas (placebo; n=89) in addition to their ventilatory support. Patients that exhibited a $PaO_2 > 60$ mm Hg and a pH < 7.55 were weaned to 5 ppm INOmax® or placebo. The primary results from the CINRGI study are presented in Table 4. ECMO was the primary endpoint of the study.

Table 1: Summary of Clinical Results from CINRGI Study

	Placebo	INOmax®	P value
Death or ECMO	51/89 (57%)	30/97 (31%)	<0.001
Death	5/89 (6%)	3/97 (3%)	0.48

[0040] Significantly fewer neonates in the ECMO group required ECMO, and INOmax® significantly improved oxygenation, as measured by PaO_2, OI, and alveolar-arterial gradient.

[0041] NINOS study. (See Inhaled Nitric Oxide in Full-Term and Nearly Full-Term Infants with Hypoxic Respiratory Failure; NEJM, Vol. 336, No. 9, 597).

[0042] The Neonatal Inhaled Nitric Oxide Study (NINOS) group conducted a double-blind, randomized, placebo-controlled, multicenter trial in 235 neonates with hypoxic respiratory failure. The objective of the study was to determine whether iNO would reduce the occurrence of death and/or initiation of ECMO in a prospectively defined cohort of term or near-term neonates with hypoxic respiratory failure unresponsive to conventional therapy. Hypoxic respiratory failure was caused by meconium aspiration syndrome (MAS; 49%), pneumonia/sepsis (21%), idiopathic primary pulmonary hypertension of the newborn (PPHN; 17%), or respiratory distress syndrome (RDS; 11%). Infants ≤ 14 days of age (mean, 1.7 days) with a mean PaO_2 of 46 mm Hg and a mean oxygenation index (OI) of 43 cm H_2O/mmHg were initially randomized to receive 100% O_2 with (n=114) or without (n=121) 20 ppm NO for up to 14 days. Response to study drug was defined as a change from baseline in PaO_2 30 minutes after starting treatment (full response = > 20 mmHg, partial = 10–20 mm Hg, no response = < 10 mm Hg). Neonates with a less than full response were evaluated for a response to 80 ppm NO or control gas. The primary results from the NINOS study are presented in Table 2.

Table 2: Summary of Clinical Results from NINOS Study

	Control (n=121)	NO (n=114)	P value
Death or ECMO*, †	77 (64%)	52 (46%)	0.006
Death	20 (17%)	16 (14%)	0.60
ECMO	66 (55%)	44 (39%)	0.014

* Extracorporeal membrane oxygenation
† Death or need for ECMO was the study's primary end point

[0043] Adverse Events from CINRGI & NINOS. Controlled studies have included 325 patients on INOmax® doses of 5 to 80 ppm and 251 patients on placebo. Total mortality in the pooled trials was 11% on placebo and 9% on INOmax®, a result adequate to exclude INOmax® mortality being more than 40% worse than placebo.

[0044] In both the NINOS and CINRGI studies, the duration of hospitalization was similar in INOmax® and placebo-treated groups.

[0045] From all controlled studies, at least 6 months of follow-up is available for 278 patients who received INOmax® and 212 patients who received placebo. Among these patients, there was no evidence of an AE of treatment on the need for re-hospitalization, special medical services, pulmonary disease, or neurological squeal.

[0046] In the NINOS study, treatment groups were similar with respect to the incidence and severity of intracranial hemorrhage, Grade IV hemorrhage, per ventricular leukomalacia, cerebral infarction, seizures requiring anticonvulsant therapy, pulmonary hemorrhage, or gastrointestinal hemorrhage.

[0047] The table below shows adverse reactions that occurred in at least 5% of patients receiving INOmax® in the CINRGI study. None of the differences in these adverse reactions were statistically significant when iNO patients were compared to patients receiving placebo.

Table 3: ADVERSE REACTIONS ON THE CINRGI TRIAL

Adverse Reaction	Placebo (n=89)	Inhaled NO (n=97)
Atelectasis	5 (4.8%)	7 (6.5%)
Bilirubinemia	6 (5.8%)	7 (6.5%)
Hypokalemia	5 (4.8%)	9 (8.3%)
Hypotension	3 (2.9%)	6 (5.6%)
Thrombocytopenia	20 (19.2%)	16 (14.8%)

[0048] Post-Marketing Experience. The following AEs have been reported as part of the post-marketing surveillance. These events have not been reported above. Given the nature of spontaneously reported post-marketing surveillance data, it is impossible to determine the actual incidence of the events or definitively establish their causal relationship to the drug. The listing is alphabetical: dose errors associated with the delivery system; headaches associated with environmental exposure of INOmax® in hospital staff; hypotension associated with acute withdrawal of the drug; hypoxemia associated with acute withdrawal of the drug; pulmonary edema in patients with CREST syndrome.

[0049] An analysis of AEs and SAEs from both the CINRGI and NINOS studies, in addition to post-marketing surveillance, did not suggest that patients who have pre-existing LVD could experience an increased risk of AEs or SAEs. Nor was it predictable to physicians skilled in the art that patients having pre-existing LVD (possibly identified as those patients having a PCWP greater than 20 mmHg) should be evaluated in view of the benefit versus risk of using iNO in patients with clinically significant LVD, and that these patients should be evaluated on a case by case basis.

EXAMPLE 1: INOT22 STUDY

[0050] The INOT22, entitled "Comparison of supplemental oxygen and nitric oxide for inhalation plus oxygen in the evaluation of the reactivity of the pulmonary vasculature during acute pulmonary vasodilatory testing" was conducted both to access the safety and effectiveness

of INOmax® as a diagnostic agent in patients undergoing assessment of pulmonary hypertension (primary endpoint), and to confirm the hypothesis that iNO is selective for the pulmonary vasculature (secondary endpoint).

[0051] During, and upon final analysis of the INOT22 study results, applicants discovered that rapidly decreasing the pulmonary vascular resistance, via the administration of iNO to a patient in need of such treatment, may be detrimental to patients with concomitant, pre-existing LVD. Therefore, a precaution for patients with LVD was proposed to be included in amended prescribing information for INOmax®. Physicians were further informed to consider reducing left ventricular afterload to minimize the occurrence of pulmonary edema in patients with pre-existing LVD.

[0052] In particular, the INOT22 protocol studied consecutive children undergoing cardiac catheterization that were prospectively enrolled at 16 centers in the US and Europe. Inclusion criteria: 4 weeks to 18 years of age, pulmonary hypertension diagnosis, i.e. either idiopathic pulmonary hypertension (IPAH) or related to congenital heart disease (CHD) (repaired or unrepaired) or cardiomyopathy, with pulmonary vascular resistance index (PVRI) > 3 u-m^2. Later amendments, as discussed herein, added an additional inclusionary criteria of a PCWP less than 20 gmm Hg. Patients were studied under general anaesthesia, or with conscious sedation, according to the practice of the investigator. Exclusion criteria: focal infiltrates on chest X-ray, history of intrinsic lung disease, and/or currently taking PDE-5 inhibitors, prostacyclin analogues or sodium nitroprusside. The study involved supplemental O_2 and NO for inhalation plus O_2 in the evaluation of the reactivity of the pulmonary vasculature during acute pulmonary vasodilator testing. Consecutive children undergoing cardiac catheterization were prospectively enrolled at 16 centers in the US and Europe. As hypotension is expected in these neonatal populations, the comparison between iNO and placebo groups is difficult to assess. A specific secondary endpoint was evaluated in study INOT22 to provide a more definitive evaluation.

[0053] The primary objective was to compare the response frequency with iNO and O_2 vs. O_2 alone; in addition, all subjects were studied with iNO alone. Patients were studied during five periods: Baseline 1, Treatment Period 1, Treatment Period 2, Baseline 2 and Treatment Period 3. All patients received all three treatments; treatment sequence was randomized by

center in blocks of 4; in Period 1, patients received either NO alone or O_2 alone, and the alternate treatment in Period 3. All patients received the iNO and O_2 combination treatment in Period 2. Once the sequence was assigned, treatment was unblinded. Each treatment was given for 10 minutes prior to obtaining hemodynamic measurements, and the Baseline Period 2 was at least 10 minutes.

[0054] Results for the intent-to-treat (ITT) population, defined as all patients who were randomized to receive drug, indicated that treatment with NO plus O_2 and O_2 alone significantly increased systemic vascular resistance index (SVRI) (Table 4). The change from baseline for NO plus O_2 was 1.4 Woods Units per meter^2 (WU·m^2) ($p = 0.007$) and that for O_2 was 1.3 WU·m^2 ($p = 0.004$). While the change from baseline in SVRI with NO alone was -0.2 WU·m^2 ($p = 0.899$) which demonstrates a lack of systemic effect.

Table 4: SVRI Change From Baseline by Treatment (Intent-to-Treat)

	Treatment		
SVRI ($WU \cdot m^2$)	**NO Plus O_2 (n=109)**	**O_2 (n=106)**	**NO (n=106)**
Baseline (room air)			
Mean	17.2	17.6	18.0
Standard Deviation (SD)	8.86	9.22	8.44
Median	15.9	16.1	16.2
Minimum, maximum	-7.6, 55.6	-7.6, 55.6	1.9, 44.8
Post-treatment			
Mean	18.7	18.9	17.8
SD	9.04	8.78	9.40
Median	17.1	17.1	15.4
Minimum, maximum	3.0, 47.4	3.9, 43.6	3.3, 50.7
Change From Baseline			
Mean	1.4	1.3	-0.2
SD	5.94	5.16	4.65
Median	1.2	1.0	0.2
Minimum, maximum	-20.5, 19.1	-18.1, 17.7	-12.5, 12.7
p-value[a]	0.007	0.004	0.899
Pairwise comparisons NO plus O_2 versus O_2, p=0.952 NO plus O_2 versus NO, p=0.014 O_2 versus NO, p=0.017			

[a] p-value from a Wilcoxon Signed Rank Test. Only patients with data to determine response at both treatments are included in this analysis.

Source: INOT22 CSR Table 6.4.1 and Appendix 16.2.6 (ATTACHMENT 1)

[0055] The ideal pulmonary vasodilator should reduce PVRI and/or PAPm while having no appreciable effect on systemic blood pressure or SVRI. In this case, the ratio of PVRI to SVRI would decrease, given some measure of the selectivity of the agent for the pulmonary vascular bed. The change in the ratio of PVRI to SVRI by treatment is shown in Table 5.

Table 5: Change in Ratio of PVRI to SVRI by Treatment (Intent-to-Treat)

	Treatment		
Ratio PVRI/SVRI	**NO Plus O_2 (n=108)**	**O_2 (n=105)**	**NO (n=106)**
Baseline			
Mean	0.6	0.5	0.6
SD	0.60	0.45	0.56
Median	0.5	0.5	0.4
Minimum, Maximum	-1.6, 4.7	-1.6, 1.8	0.0, 4.7
Post Treatment			
Mean	0.4	0.4	0.5
SD	0.31	0.31	0.46
Median	0.3	0.4	0.3
Minimum, Maximum	0.0, 1.3	0.0, 1.4	-1.2, 2.2
Change from Baseline			
Mean	-0.2	-0.1	-0.1
SD	0.52	0.31	0.54
Median	-0.1	-0.1	0.0
Minimum, Maximum	-4.4, 2.0	-1.6, 2.0	-4.4, 1.6
P Value[1]	< 0.001	< 0.001	0.002

[1] Wilcoxon Signed Rank Test
Source: INOT22 CSR Table 6.5.1 (ATTACHMENT 2)

[0056] All three treatments have a preferential effect on the pulmonary vascular bed, suggesting that all three are selective pulmonary vasodilators. The greatest reduction in the ratio was during treatment with NO plus O_2, possibly due to the decrease in SVRI effects seen with O_2 and NO plus O_2. These results are displayed as percent change in the ratio (See Table 6).

Table 6: Percent Change in Ratio of PVRI to SVRI by Treatment (Intent-to-Treat)

	Treatment		
Ratio PVRI/SVRI	**NO Plus O_2 (n=108)**	**O_2 (n=105)**	**NO (n=106)**
Baseline			
Mean	0.6	0.5	0.6
SD	0.60	0.45	0.56
Median	0.5	0.5	0.4
Minimum, Maximum	-1.6, 4.7	-1.6, 1.8	0.0, 4.7
Post Treatment			
Mean	0.4	0.4	0.5
SD	0.31	0.31	0.46
Median	0.3	0.4	0.3
Minimum, Maximum	0.0, 1.3	0.0, 1.4	-1.2, 2.2
Percent Change from Baseline			
Mean	-33.5	-19.3	-6.2
SD	36.11	34.59	64.04
Median	-34.0	-21.3	-13.8
Minimum, Maximum	-122.2, 140.1	-122.7, 93.3	-256.1, 294.1
P Value[1]	< 0.001	< 0.001	0.006

[1] Wilcoxon Signed Rank Test
Source: INOT22 CSR Table 6.5.2 (ATTACHMENT 3)

[0057] NO plus O_2 appeared to provide the greatest reduction in the ratio, suggesting that NO plus O_2 was more selective for the pulmonary vasculature than either agent alone.

[0058] Overview of Cardiovascular Safety. In the INOT22 diagnostic study, all treatments (NO plus O_2, O_2, and NO) were well-tolerated. Seven patients of 134 treated experienced an AE during the study. These included cardiac arrest, bradycardia, low cardiac output (CO) syndrome, elevated ST segment (the portion of an electrocardiogram between the end of the QRS complex and the beginning of the T wave) on the electrocardiography (ECG)

decreased O_2 saturation, hypotension, mouth hemorrhage and pulmonary hypertension (PH). The numbers of patients and events were too small to determine whether risk for AEs differed by treatment, diagnosis, age, gender or race. Eight patients are shown in Table 5 due to the time period in which events are reported. AEs were reported for 12 hours or until hospital discharge (which limits the period in which such events can be reported). There is technically no time limit in which SAEs are to be reported. So, there were 7 AEs during the study and at least one SAE after the study.

[0059] A total of 4 patients had AEs assessed as being related to study drug. These events included bradycardia, low CO syndrome, ST segment elevation on the ECG, low O_2 saturation, PH and hypotension. All but 2 AEs were mild or moderate in intensity and were resolved. Study treatments had slight and non-clinically significant effects on vital signs including heart rate, systolic arterial pressure and diastolic arterial pressure. When an investigator records an AE, they are required to say if (in their opinion) the event is related to the treatment or not. In this case, 4 of 7 were considered by the investigator to be related to treatment.

[0060] The upper limit of normal PCWP in children is 10-12 mm Hg and 15 mm Hg in adults. In INOT22, a baseline PCWP value was not included as exclusion criteria. However, after the surprising and unexpected identification of SAEs in the early tested patients, it was determined that patients with pre-existing LVD had an increased risk of experiencing an AE or SAE upon administration (e.g., worsening of left ventricular function due to the increased flow of blood through the lungs). Accordingly, the protocol for INOT22 was thereafter amended to exclude patients with a baseline PCWP greater than 20 mm Hg after one patient experienced acute circulatory collapse and died during the study. The value "20 mm Hg" was selected to avoid enrollment of a pediatric population with LVD such that they would be most likely at-risk for these SAEs.

[0061] SAEs were collected from the start of study treatment until hospital discharge or 12 hours, whichever occurred sooner. Three SAEs were reported during the study period, and a total of 7 SAEs were reported. Three of these were fatal SAEs and 4 were nonfatal (one of which led to study discontinuation). In addition, one non-serious AE also lead to

discontinuation. A list of subjects who died, discontinued or experienced an SAE is provided in Table 5 below.

Table 5: Subjects that died, discontinued or experienced SAEs

Patient number	AE	Serious?	Fatal?	Discontinued treatment?
01020	Desaturation (hypoxia)	No	No	Yes
02002	Pulmonary edema	Yes	No	No
04001	Hypotension and cardiac arrest	Yes	Yes	No
04003	Hypotension and ECG changes	Yes	No	Yes
04008	Hypotension and hypoxemia	Yes	Yes	No
05002	Hypoxia and bradycardia (also pulmonary edema)	Yes	Yes	No
07003	Cardiac arrest	Yes	No	No
17001	Hypoxia	Yes	No	No

[0062] Two of the 3 fatal SAEs were deemed related to therapy. All 4 non-fatal SAEs were also considered related to therapy. The numbers of patients and events were too small to determine whether risk for SAEs differed by treatment, diagnosis, age, gender or race. At least two patients developed signs of pulmonary edema (subjects 05002 and 02002). This is of interest because pulmonary edema has previously been reported with the use of iNO in patients with LVD, and may be related to decreasing PVRI and overfilling of the left atrium. (Hayward CS et al., 1996, Inhaled Nitric Oxide in Cardiac Failure: Vascular Versus Ventricular Effects, *J Cardiovascular Pharmacology* 27:80-85; Bocchi EA et al., 1994, Inhaled Nitric Oxide Leading to Pulmonary Edema in Stable Severe Heart Failure, *Am J Cardiology* 74:70-72; and, Semigran MJ et al., 1994, Hemodynamic Effects of Inhaled Nitric Oxide in Heart Failure, *J Am Coll Cardiology* 24:982-988).

[0063] Although the SAE rate is within range for this population, it appears that patients with the most elevated PCWP at baseline had a disproportionately high number of these events. (Bocchi EA et al., 1994; Semigran MJ et al., 1994).

[0064] In the INOT22 study, 10 of the total 134 patients had a baseline PCWP $\geq$ 18 mm Hg (7.5%), of which, 3 subjects (04001, 02002 and 04003) had a SAE or were prematurely discontinued from the study (30%) compared to 6.5% for the entire cohort.

[0065] Although there were very few significant AEs in the INOT22 study, these events are consistent with the expected physiologic changes in patients with severe LVD. The events also corroborate prior observations that iNO is rapidly acting, selective for the pulmonary vasculature, and well-tolerated in most patients. The actual incidence of acute LVD during acute ventricular failure (AVT) is unknown. However, it is reasonable to expect that a significant number of patients are at-risk for an increased incidence of SAEs upon iNO treatment based upon the nature of the underlying nature of the illness, i.e., pulmonary hypertension and cardiovascular disease more generally. Thus, it would be advantageous to have physicians identify these patients prior to beginning iNO treatment, so that the physicians are alerted to this possible outcome.

[0066] Benefits and Risks Conclusions. The INOT22 study was designed to demonstrate the physiologic effects of iNO in a well defined cohort of children (i.e., intended patient population) with pulmonary hypertension using a high concentration, 80 ppm, of iNO, i.e., one that would be expected to have the maximal pharmacodynamic effect. INOT22 was the largest and most rigorous pharmacodynamic study of iNO conducted to date, and it confirms a number of prior observations, such as iNO being rapidly acting, selective for the pulmonary vasculature, and well-tolerated in most patients.

[0067] It is also acknowledged that rapidly decreasing the PVR may be undesirable and even dangerous in patients with concomitant LVD. In the INOT22 study, the overall numbers of SAEs and fatal SAEs are within the expected range for patients with this degree of cardiopulmonary disease. The overall rate is 7/124 (5.6%), which is closely comparable to the rate of 6% recently reported in a very similar cohort of patients. (Taylor CJ et al., 2007, Risk of cardiac catheterization under anaesthesia in children with pulmonary hypertension, *Br J Anaesth* 98(5):657-61). Thus, the overall rate of SAEs would seem to be more closely related to the underlying severity of illness of the patients rather than to the treatments given during this study.

[0068] The INOT22 study results demonstrate that patients who had pre-existing LVD may experience an increased rate of SAEs (e.g., pulmonary edema). During the course of the study, the protocol was amended to exclude patients with a PCWP > 20 mmHg. The benefit/risk of using iNO in patients with clinically significant LVD should be evaluated on a case by case

basis. A reduction in left ventricular afterload may perhaps be applied to minimize the occurrence of pulmonary edema.

CLAIMS

We Claim:

1. A method of reducing one or more adverse events or serious adverse events in an intended patient population comprising neonates or near-term neonates in need of being treated with inhalation of nitric oxide comprising excluding from such treatment anyone in the intended patient population having pre-existing left ventricular dysfunction.

2. The method of claim 1, wherein anyone in the intended patient population further has a pulmonary capillary wedge pressure greater than 20 mm Hg.

3. The method of claim 1, wherein the treatment further comprises inhalation of oxygen.

4. The method of claim 1, wherein the treatment is delivered using a ventilator.

5. The method of claim 1, wherein anyone in the intended patient population having pre-existing left ventricular dysfunction also having one or more conditions selected from diastolic dysfunction, hypertensive cardiomyopathy, systolic dysfunction, ischemic cardiomyopathy, viral cardiomyopathy, idiopathic cardiomyopathy, autoimmune disease related cardiomyopathy, drug-related cardiomyopathy, toxin-related cardiomyopathy, structural heart disease, valvular heart disease, congenital heart disease, idiopathic pulmonary arterial hypertension and pulmonary hypertension cardiomyopathy, and associations thereof.

6. The method of claim 1, wherein the intended patient population are at risk of one or more adverse events or serious adverse events selected from pulmonary edema, hypotension, cardiac arrest, electrocardiogram changes, hypoxemia, hypoxia and bradycardia, and, associations thereof.

7. The method of claim 1, further comprising reducing left ventricular afterload to minimize or reduce the risk of the occurrence of an adverse event or serious adverse event being pulmonary edema.

8. The method of claim 7, wherein the left ventricular afterload is minimized or reduced by administering a pharmaceutical dosage form comprising nitroglycerin or calcium channel blocker.

9. The method of claim 7, wherein the left ventricular afterload is minimized or reduced using an intra-aortic balloon pump.

10. The method of claim 1, wherein the intended patient population in need of being treated with the inhalation of nitric oxide has one or more of idiopathic pulmonary arterial hypertension characterized by PAPm > 25 mm Hg at rest, PCWP $\leq$ 15 mm Hg, and, a PVRI > 3 u·m^2; congenital heart disease with pulmonary hypertension repaired and unrepaired characterized by PAPm > 25 mm Hg at rest and PVRI > 3 u·m^2; cardiomyopathy characterized by PAPm > 25 mm Hg at rest and PVRI > 3 u·m^2 ; or, the patient is scheduled to undergo right heart catheterization to assess pulmonary vasoreactivity by acute pulmonary vasodilation testing.

11. A method of reducing the risk or preventing the occurrence, in a patient being a neonate or near-term neonate of one or more adverse events or serious adverse events associated with a medical treatment comprising inhalation of nitric oxide, said method comprising:

a. identifying a patient eligible for inhalation of nitric oxide treatment;

b. determining if said patient has pre-existing left ventricular dysfunction; and,

c. administering said medical treatment if said patient does not have pre-existing left ventricular dysfunction;

thereby reducing the risk or preventing the occurrence of the adverse event or serious adverse event associated with said medical treatment.

12. The method of claim 11, wherein said patient further exhibits a pulmonary capillary wedge pressure greater than 20 mm Hg.

13. The method of claim 11, wherein the patient who has pre-existing left ventricular dysfunction has one or more conditions selected from diastolic dysfunction, hypertensive cardiomyopathy, systolic dysfunction, ischemic cardiomyopathy, viral cardiomyopathy, idiopathic cardiomyopathy, autoimmune disease related cardiomyopathy, side effects due to drug-related cardiomyopathy, side effects due to toxin-related cardiomyopathy, structural heart disease, valvular heart disease, congenital heart disease, idiopathic pulmonary arterial hypertension, pulmonary hypertension and cardiomyopathy, and associations thereof.

14. The method of claim 12, wherein the patient who has pre-existing left ventricular dysfunction has one or more conditions selected from diastolic dysfunction, hypertensive cardiomyopathy, systolic dysfunction, ischemic cardiomyopathy, viral cardiomyopathy, idiopathic cardiomyopathy, autoimmune disease related cardiomyopathy, side effects due to drug-related cardiomyopathy, side effects due to toxin-related cardiomyopathy, structural heart disease, valvular heart disease, congenital heart disease, idiopathic pulmonary arterial hypertension, pulmonary hypertension and cardiomyopathy, or associations thereof.

15. The method of claim 11, wherein the medical treatment further comprises inhalation of oxygen.

16. The method of claim 11, wherein the treatment is delivered using a ventilator.

17. The method of claim 11, further comprising reducing left ventricular afterload to minimize or reduce the risk of the occurrence of an adverse event or serious adverse event being pulmonary edema.

18. The method of claim 17, wherein the left ventricular afterload is minimized or reduced by administering a pharmaceutical dosage form comprising nitroglycerin or calcium channel blocker.

19. The method of claim 17, wherein the left ventricular afterload is minimized or reduced using an intra-aortic balloon pump.

ABSTRACT

The invention relates methods of reducing the risk or preventing the occurrence of an adverse event (AE) or a serious adverse event (SAE) associated with a medical treatment comprising inhalation of nitric oxide.

Application Data Sheet

Application Information

Application number:	
Filing Date:	
Application Type:	Continuation
Subject Matter:	Utility
Suggested classification & subclass:	600/481 (surgery/cardiovascular)
Suggested Group Art Unit:	
CD-ROM or CD-R:	None
Number of CD disks:	
Number of copies of CDs:	
Sequence submission::	
Computer Readable Form (CRF):	
Number of copies of CRF:	
Title:	METHODS OF TREATING TERM AND NEAR-TERM NEONATES HAVING HYPOXIC RESPIRATORY FAILURE ASSOCIATED WITH CLINICAL OR ECHOCARDIOGRAPHIC EVIDENCE OF PULMONARY HYPERTENSION
Attorney Docket Number:	I001-0002USC1
Request for Early Publication:	NO
Request for Non-Publication:	NO
Suggested Drawing Figure:	NA
Total Drawing Sheets:	NA
Small Entity:	YES
Latin name:	
Variety denomination name:	
Petition included:	Yes
Petition Type:	Accelerated Examination

Licensed US Govt. Agency:
Contract or Grant Numbers:
Secrecy Order in Parent Appl:

Applicant Information

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Correspondence Information

Correspondence Customer Number:: 49584

Representative Information

Representative Customer Number:: 49584

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This application	continuation of	12/494,598	06/30/2009

Foreign Priority Information

Country::	Application number::	Filing Date::	Priority Claimed::

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Doc Code: PET.SPRE.ACX
Doc Description: Petition for 12-month Accelerated Exam

PTO/SB/28 (07-09)
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PETITION TO MAKE SPECIAL UNDER ACCELERATED EXAMINATION PROGRAM

Attorney Docket Number	I001-0002USC1	First Named Inventor	James S. Baldassarre
Application Number (if Known)			
Title of Invention	Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension		

APPLICANT HEREBY PETITIONS TO MAKE THE ABOVE-IDENTIFIED APPLICATION SPECIAL UNDER THE REVISED ACCELERATED EXAMINATION PROGRAM. See Instruction sheet on page 3.

1. **Claims of the application:**
   a. The application must contain three (3) or fewer independent claims and twenty (20) or fewer total claims. The application may not contain any multiple dependent claims.

   b. **Applicant hereby agrees not to separately argue the patentability of any dependent claim during any appeal** in the application. Specifically, the applicant agrees that the dependent claims will be grouped together with and not argued separately from the independent claim from which they depend in any appeal brief filed in the application (37 CFR 41.37(c)(1)(vii)).

   c. The claims must be directed to **a single invention**.

2. **Interviews:**
   Applicant hereby agrees to have (if requested by examiner):
   a. An interview (including an interview before a first Office action) to discuss the prior art and any potential rejections or objections with the intention of clarifying and possibly resolving all issues with respect to patentability at that time, and

   b. A telephonic interview to make an election without traverse if the Office determines that the claims are not obviously directed to a single invention.

3. **Preexamination Search Statement and Accelerated Examination Support Document:**
   With this petition, applicant is providing: a **preexamination search statement**, in compliance with the requirements set forth in item 8 of the instruction sheet, and an **"accelerated examination support document"** that includes:
   a. An **information disclosure statement** in compliance with 37 CFR 1.98 citing each reference deemed most closely related to the subject matter of each of the claims;

   b. For each reference cited, **an identification of all the limitations of the claims** that are disclosed by the reference specifying where the limitation is disclosed in the cited reference;

   c. A **detailed explanation of how each of the claims are patentable** over the references cited with the particularity required by 37 CFR 1.111(b) and (c);

   d. A concise **statement of the utility** of the invention as defined in each of the independent claims (unless the application is a design application);

   e. An identification of any cited references that may be disqualified as prior art under 35 U.S.C. 103(c) as amended by the CREATE act; and

   f. **A showing of where each limitation of the claims finds support under the first paragraph of 35 U.S.C. 112** in the written description of the specification. If applicable, the showing must also identify: (1) each means- (or step-) plus-function claim element that invokes consideration under 35 U.S.C. 112, ¶6; and (2) the structure, material, or acts that correspond to any means- (or step-) plus-function claim element that invokes consideration under 35 U.S.C. 112, ¶6. If the application claims the benefit of one or more applications under title 35, United St ates Code, the showing must also include where each limitation of the claims finds support under the first paragraph of 35 U.S.C. 112 in each such application in which such support exists.

Doc Code: PET.SPRE.ACX
Doc Description: Petition for 12-month Accelerated Exam

PTO/SB/28 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U. S. DEPARTMENT OF COMMERCE

PETITION TO MAKE SPECIAL UNDER ACCELERATED EXAMINATION PROGRAM (Continued)

Attorney Docket Number	I001-0002USC1	First Named Inventor	James S. Baldassarre

Attachments:

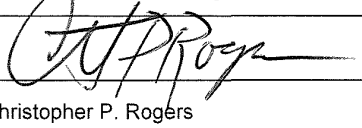
a.	X	Accelerated Examination Support Document (see item 3 above).
b.	X	A statement, in compliance with the requirements set forth in item 8 of the instruction sheet, detailing the preexamination search which was conducted.
c.	X	Information Disclosure Statement.
d.	☐	Other (*e.g.*, a statement that the claimed subject matter is directed to environmental quality, energy, or countering terrorism (37 CFR 1.102(c)(2)).

Fees: The following fees must be filed electronically via EFS or EFS-Web:

a.	The basic filing fee, search fee, examination fee, and application size fee (if required) under 37 CFR 1.16.
b.	Petition fee under 37 CFR 1.17(h) - unless the petition is filed with a showing under 37 CFR 1.102(c)(2).

Signature:

Click Remove if you wish to remove this signatory — Remove

Signature		Date	21 June 2010
Name (Print/Typed)	Christopher P. Rogers	Registration Number	36334

Click Add if you wish to add additional signatory — Add

Note: *Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required in accordance with 37 CFR 1.33 and 10.18. Please see 37 CFR 1.4(d) for the form of the signature.*

Electronic Patent Application Fee Transmittal

Application Number:	12820866
Filing Date:	
Title of Invention:	METHODS OF TREATING TERM AND NEAR-TERM NEONATES HAVING HYPOXIC RESPIRATORY FAILURE ASSOCIATED WITH CLINICAL OR ECHOCARDIOGRAPHIC EVIDENCE OF PULMONARY HYPERTENSION
First Named Inventor/Applicant Name:	James S. Baldassarre
Filer:	Daniel Leo Hayes/Anna Goforth
Attorney Docket Number:	I001-0002USC1

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Petition fee- 37 CFR 1.17(h) (Group III)	1464	1	130	130
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**130**

Electronic Acknowledgement Receipt

EFS ID:	7869464
Application Number:	12820866
International Application Number:	
Confirmation Number:	2913
Title of Invention:	METHODS OF TREATING TERM AND NEAR-TERM NEONATES HAVING HYPOXIC RESPIRATORY FAILURE ASSOCIATED WITH CLINICAL OR ECHOCARDIOGRAPHIC EVIDENCE OF PULMONARY HYPERTENSION
First Named Inventor/Applicant Name:	James S. Baldassarre
Customer Number:	49584
Filer:	Daniel Leo Hayes/Anna Goforth
Filer Authorized By:	Daniel Leo Hayes
Attorney Docket Number:	I001-0002USC1
Receipt Date:	22-JUN-2010
Filing Date:	
Time Stamp:	17:41:41
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Petition for 12-month Accelerated Exam	MV7371.PDF	159348 9e6e0c175f9993ed261943de1743ba052eaa965b	no	2
Warnings:					
This is not a USPTO supplied Accelerated Exam SB28 form.					
Information:					
2	Fee Worksheet (PTO-875)	fee-info.pdf	30303 88a9749b185f475ef6662a53e93dca2680fae104	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	189651		

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<u>New Applications Under 35 U.S.C. 111</u>
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (12-04)

Date 06/22/10

Approved for use through 7/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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PATENT APPLICATION FEE DETERMINATION RECORD
Substitute for Form PTO-875

Application or Docket Number
12/820,866

APPLICATION AS FILED – PART I

127878875

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)	OR	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	82		N/A	
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	270		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	110		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	19 minus 20 =		x$26		OR	x$52	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =	*	x$110			x$220	
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $270 ($135 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR						
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))			195			390	
			TOTAL	462		TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED – PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDI-TIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDI-TIONAL FEE ($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					N/A		OR	N/A	
						TOTAL ADD'T FEE		OR	TOTAL ADD'T FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDI-TIONAL FEE ($)	OR	RATE ($)	ADDI-TIONAL FEE ($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					N/A		OR	N/A	
						TOTAL ADD'T FEE		OR	TOTAL ADD'T FEE	

* If the entry in column 1 is less than the entry in column 2; write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
12/820,866	06/22/2010	1614	462	I001-0002USC1	19	2

CONFIRMATION NO. 2913

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OC000000042277526

Date Mailed: 06/24/2010

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

James S. Baldassarre, Doylestown, PA;
Ralf Rosskamp, Chester, NJ;

Assignment For Published Patent Application

Ikaria Holdings, Inc., Clinton, NJ

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 12/494,598 06/30/2009

Foreign Applications

If Required, Foreign Filing License Granted: 06/24/2010

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 12/820,866**

Projected Publication Date: 12/30/2010

Non-Publication Request: No

Early Publication Request: No
** **SMALL ENTITY** **

Title

METHODS OF TREATING TERM AND NEAR-TERM NEONATES HAVING HYPOXIC RESPIRATORY FAILURE ASSOCIATED WITH CLINICAL OR ECHOCARDIOGRAPHIC EVIDENCE OF PULMONARY HYPERTENSION

Preliminary Class

514

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/820,866	06/22/2010	James S. Baldassarre	1001-0002USC1	2913

49584 7590 07/08/2010

LEE & HAYES, PLLC
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EXAMINER	
ARNOLD, ERNST V	
ART UNIT	**PAPER NUMBER**
1616	

MAIL DATE	DELIVERY MODE
07/08/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

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JUL 0 8 2010

LEE & HAYES, PLLC
601 W. RIVERSIDE AVENUE
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SPOKANE WA 99201

In re Application of: BALDASSARRE et al.
Serial No.: 12/820,866

Filed: June 22, 2010
Docket: I001-0002USC1
Title: **METHODS OF TREATING TERM AND NEAR-TERM NEONATES HAVING HYPOXIC RESPIRATORY FAILURE ASSOCIATED WITH CLINICAL OR ECHOCARDIOGRAPHIC EVIDENCE OF PULMONARY HYPERTENSION**

DECISION ON PETITION TO MAKE SPECIAL FOR NEW APPLICATION UNDER 37 C.F.R. § 1.102 & M.P.E.P. § 708.02

This is a decision on the petition filed on June 22, 2010 to make the above-identified application special for accelerated examination procedure under 37 C.F.R. § 1.102(d).

The petition to make the application special is GRANTED.

The application is eligible for accelerated examination and the petition complies with the conditions for granting the application special status pursuant to the "Change to Practice for Petitions in Patent Applications to Make Special and for Accelerated Examination" published June 26, 2006, in the Federal Register. (71 Fed. Reg. 36323).

The prosecution of the instant application will be conducted expeditiously according to the following guidelines.

1. The application will be docketed to an examiner and taken up for action within two weeks of the date of this decision.

2. Restriction Practice:
   If the examiner determines that the claims are not directed to a single invention, a telephone request to elect one single invention will be made pursuant to MPEP 812.01. As a prerequisite to the grant of this petition, the applicant has agreed to make an oral election, by telephone, without traverse. If the applicant refuses to make an election

without traverse, or the examiner cannot reach the applicant after a reasonable effort, the examiner will treat the first claimed invention (invention defined by claim 1) as having being constructively elected without traverse for examination.

3. Office action:
If it is determined that, after appropriate consultation, there is a potential rejection or any other issue to be addressed, the examiner will telephone the applicant and arrange an interview to discuss and resolve the issue. An Office action, other than a Notice of Allowance and Fee(s) Due (Notice of Allowance), will not be issued unless either: 1) an interview was conducted but did not result in agreed to action that places the application in condition for allowance, or, 2) a determination is made that an interview would be unlikely to result in the application being placed in condition for allowance, and 3) an internal conference has been held to review any rejection of any claim.

4. Time for Reply:
An Office action other than a Notice of Allowance or a final Office action will set a shortened statutory period of one month or thirty days, whichever is longer, for reply with no extension of time available under 37 CFR 1.136(a). Failure to timely file a reply within this non-extendible period for reply will result in the abandonment of the application.

5. Reply by Applicant:
A timely reply to an Office action other than the Notice of Allowance must be submitted electronically via EFS or EFS-web and limited to addressing the rejections, objections and requirement made. Any amendment that attempts to: 1) add claims which would result in more than three pending independent claims or more than twenty pending total claims; 2) present claims not encompassed by the pre-examination search or an updated accelerated examination support document; or 3) present claims that are directed a non-elected invention or an invention other than that previously claimed and examined in the application, will be treated as not fully responsive and will not be entered.

For any amendment to the claims (including any new claim) that is not encompassed by the accelerated examination support document, applicant must provide an updated accelerated examination support document that encompasses the amended or new claims at the time of filing of the amendment.

To proceed expeditiously with the examination, it is recommended that a reply with amendments made to any claim or with any new claim being added be accompanied by an updated accelerated examination support document or a statement explaining how the amended or new claim is supported by the original accelerated examination support document.

6. Information Disclosure Statement (IDS):
Any IDS filed during prosecution must be submitted electronically via EFS or EFS-web, accompanied by an updated accelerated examination support document, and be in compliance with 37 CFR 1.97 and 1.98.

7. Post-Allowance Processing:
   To expedite processing of the allowed application into a patent, the applicant must: 1) pay the required fees within one month of the date of the Notice of Allowance, and 2) not file any post allowance papers not required by the Office. In no event may the issue fee be paid and accepted later than three months from the date of the Notice of Allowance.

8. After-Final and Appeal Procedures:
   To expedite prosecution, after receiving the final Office action, applicant must: 1) promptly file a notice of appeal, an appeal brief and appeal fees; and 2) not request a pre-appeal brief conference.

   Any amendment, affidavit or other evidence filed after final Office action must comply with applicable rules and the requirements outlined in numbered paragraphs 5 and 6 above.

   On appeal, the application will proceed according to normal appeal procedures. After appeal, the application will again be treated special.

9. Proceedings Outside the Normal Examination Process:
   If the application becomes involved in a proceeding that is outside the normal examination process (e.g., a secrecy order, national security review, interference proceeding, petitions under 37 CFR 1.181, 182 or 183), the application will be treated special before and after such proceeding.

10. Final Disposition:
    The twelve month goal of this accelerated examination procedure ends with a final disposition. The mailing of a final Office action, a Notice of Allowance, the filing of a Notice of Appeal, or the filing of a Request for Continued Examination (RCE) is the final disposition.

If, during prosecution, a paper is not filed electronically using EFS-web, a reply is filed but is not fully responsive, the application is involved in an appeal, or a proceeding outside normal examination process, the application will still be examined expeditiously, however, the final disposition may occur more than twelve months from the filing of the application.

Any inquiry regarding this decision should be directed to Michael P. Woodward, Quality Assurance Specialist, at (571) 272-8373.

/MP Woodward/
Michael P. Woodward, Quality Assurance Specialist
Technology Center 1600

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/820,866	06/22/2010	James S. Baldassarre	I001-0002USC1	2913

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EXAMINER	
ARNOLD, ERNST V	
ART UNIT	**PAPER NUMBER**
1613	
MAIL DATE	**DELIVERY MODE**
09/23/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary for Applications Under Accelerated Examination	**Application No.** 12/820,866	**Applicant(s)** BALDASSARRE ET AL.	
	Examiner ERNST V. ARNOLD	**Art Unit** 1616	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Since this application has been granted special status under the accelerated examination program,

NO extensions of time under 37 CFR 1.136(a) will be permitted and a SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE:

ONE MONTH OR THIRTY (30) DAYS, WHICHEVER IS LONGER,
FROM THE MAILING DATE OF THIS COMMUNICATION – if this is a non-final action or a *Quayle* action.
(Examiner: For **FINAL** actions, please use PTOL-326.)

The objective of the accelerated examination program is to complete the examination of an application within twelve months from the filing date of the application. Any reply must be filed electronically via EFS-Web so that the papers will be expeditiously processed and considered. If the reply is not filed electronically via EFS-Web, the final disposition of the application may occur later than twelve months from the filing of the application.

Status

1)☐ Responsive to communication(s) filed on _____.

2)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

3)☒ Claim(s) *1-19* is/are pending in the application.

3a) Of the above claim(s) _____ is/are withdrawn from consideration.

4)☐ Claim(s) _____ is/are allowed.

5)☒ Claim(s) *1-19* is/are rejected.

6)☐ Claim(s) _____ is/are objected to.

7)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

8)☐ The specification is objected to by the Examiner.

9)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

10)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

11)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

- See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *6/22/10*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Claims 1-19 are pending and under examination.

Comment: After an internal conference it was determined that an interview would be unlikely to place the application in condition for allowance.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 1-19 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The term "serious" in claims 1, 7, 11 and 17 is a relative term which renders the claim indefinite. The term "serious" is not defined by the claim, the specification does not provide a standard for ascertaining the requisite degree, and one of ordinary skill in the art would not be reasonably apprised of the scope of the invention. Furthermore, any adverse effect in a neonate could be considered serious and thus it is redundant. The specification attempts to define the terms in [0025] and [0027] where the “adverse event” is any unfavorable and unintended sign, symptom, or disease temporarily associated with the use of a medicinal/investigational product and is considered clinically significant and “serious adverse event is a significant hazard or side effect which would then make it clinically significant and no different from an “adverse event”. Claims 2-20 and 12-19 are rejected as being indefinite because they are dependent on indefinite base claims.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 5, 6, 13 and 14 rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Claims 5, 6, 13 and 14 recite: "associations thereof". It is unclear what the metes and bounds of "associations thereof" might encompass as it is unknown what might be associated with the conditions listed. The American Illustrated Medical Dictionary (Dorland, 1914, 7th Ed, page 113) defines "association" as: "The coordination of the functions of similar part." It is unclear what parts are intended to be coordinated by Applicant with "associations thereof". The Examiner suggests deleting the term.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 11-19 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for reducing the risk in a patient of one or more adverse events associated with a medical treatment comprising inhalation of nitric oxide, does not reasonably provide enablement for *a method of preventing the occurrence in a patient of one or more adverse events associated with a medical treatment comprising inhalation of nitric oxide*. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly

connected, to make and use the invention commensurate in scope with these claims without an undue amount of experimentation.

Let the Examiner be clear: Applicant is not enabled for *a method of preventing the occurrence in a patient of one or more adverse events associated with a medical treatment comprising inhalation of nitric oxide.*

The factors to be considered in determining whether a disclosure meets the enablement requirement of 35 U.S.C. 112, first paragraph, have been described in *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988). Among these factors are: 1) scope or breadth of the claims; 2) nature of the invention; 3) relative level of skill possessed by one of ordinary skill in the art; 4) state of, or the amount of knowledge in, the prior art; 5) level or degree of predictability, or a lack thereof, in the art; 6) amount of guidance or direction provided by the inventor; 7) presence or absence of working examples; and 8) quantity of experimentation required to make and use the claimed invention based upon the content of the supporting disclosure. When the above factors are weighed, it is the Examiner's position that one skilled in the art could not practice the invention without undue experimentation. While all of the factors have been considered, only those required for a *prima facie* case are set forth below.

1) Scope or breadth of the claims

The claims are broader in scope than the enabling disclosure. The specification merely discloses, without more, methods of reducing the risk of adverse events associated with a medical treatment comprising inhalation of nitric oxide. However, Applicant is purporting to prevent adverse events associated with a medical treatment comprising inhalation of nitric oxide.

2) Nature of the invention

The nature of the invention is directed to methods of reducing the risk of adverse events associated with a medical treatment comprising inhalation of nitric oxide.

3) Relative level of skill possessed by one of ordinary skill in the art

MPEP 2141.03 states (in part), "A person of ordinary skill in the art is also a person of ordinary creativity, not an automaton." KSR International Co. v. Teleflex Inc., 127 S.Ct. 1727, 167 LEd2d 705, 82 USPQ2d 1385, 1397 (2007). "[I]n many cases a person of ordinary skill will be able to fit the teachings of multiple patents together like pieces of a puzzle." Id. Office personnel may also take into account "the inferences and creative steps that a person of ordinary skill in the art would employ." Id. At 1396, 82 USPQ2d at 1396. The "hypothetical person having ordinary skill in the art' to which the claimed subject matter pertains would, of necessity have the capability of understanding the scientific and engineering principles applicable to the pertinent art." Ex parte Hiyamizu, 10 USPQ2d 1393, 1394 (Bd. Pat. App. & Inter. 1988) (The Board disagreed with the examiner's definition of one of ordinary skill in the art (a doctorate level engineer or scientist working at least 40 hours per week in semiconductor research or development), finding that the hypothetical person is not definable by way of credentials, and that the evidence in the application did not support the conclusion that such a person would require a doctorate or equivalent knowledge in science or engineering.).

4) State of, or the amount of knowledge in, the prior art

Beghetti et al. teach that: "Inhaled nitric oxide should be administered with caution to babies with LV dysfunction because pulmonary vasoconstriction may act as a protective mechanism of LV overfilling." (Journal of Pediatrics, 1997, page 844).

Macrae et al. (Intensive Care Med 2004, 30, pp 372-380) teach inhaled nitric oxide therapy in neonates and children and has been performed since 1992 (title and Abstract). Macrae et al. teach using echocardiography to exclude congenital heart disease as a cause of hypoxaemia prior to exposure to iNO and that inhaled NO exposure may even be harmful in some babies with congenital heart disease such as those with severe left ventricular dysfunction (page 373, bottom right to page 374, top left).

Atz et al. (Seminars in Perinatology 1997, 21(5), pp 441-455) teach inhaled nitric oxide in the neonate with cardiac disease (title). Atz et al. teach that: "Caution should be exercised when administering NO to patients with severe left ventricular dysfunction and pulmonary hypertension." (page 452, left column). Atz et al. continues with: "Therefore, in newborns with severe left ventricular dysfunction, predominantly left to right shunting at the foramen ovale and exclusively right to left shunting at the ductus arteriosus, ***NO should be used with extreme caution, if at all***. We and others have reported adverse outcomes in this circumstance." (page 452, left column) (Examiner added emphasis).

Loh et al. (Circulation 1994, 90, 2780-2785) teach that inhaled nitric oxide in patients with left ventricular dysfunction may have adverse effects in patients with LV failure (Title and Abstract).

Kinsella et al. (The Lancet 1999, 354, pp 1061-1065) teach excluding patients (premature neonates) from inhaled nitric oxide treatment if they have fatal congenital anomalies or congenital heart disease (Abstract and page 1062, Methods).

Wessel et al. (Pediatrics, 1997, 100(5), 7 pages) teaches exclusion of patients (newborns) with congenital heart disease or right ventricular dysfunction from treatment with inhaled NO

(Abstract and page 2 of 7, Methods). A patient with left ventricular dysfunction who received treatment died of an intracranial hemorrhage (page 3 of 7, left column to top right column).

5) Level or degree of predictability, or a lack thereof, in the art

Macrae et al. teach that: "Sufficient data are lacking for evaluation of the possible effets of iNO on periventricular haemorrhage or on long-term neurodevelopmental outcome." (page 377, left column).

Applicant acknowledges that this art is "unpredictable science which is also not well understood" (page 19 of 21 in the Detailed Explanation of Patentability of the Accelerated Examination Support Document filed 6/22/10).

6) Amount of guidance or direction provided by the inventor

Applicant was required to provide in the specification additional guidance and direction with respect to how use the claimed subject matter in order for the application to be enabled with respect to the full scope of the claimed invention. Although the instant specification discloses that methods of reducing the risk of adverse events associated with a medical treatment comprising inhalation of nitric oxide, it remains silent on preventing those adverse events.

7) Presence or absence of working examples

The specification fails to provide scientific data and working embodiments with respect to prevention of the adverse effects.

8) Quantity of experimentation required to make and use the claimed invention based upon the content of the supporting disclosure

One of ordinary skill in the art would have to conduct a myriad number of experiments comprising trial and error administration of NO gas to babies without a clue as to how this will

affect future development of the child such as on the long-term neurodevelopmental outcome. This is especially difficult when Applicant's own definition of 'adverse effects' embraces essentially any effect under the sun. The result of failure is potentially death of the patient. Essentially, one of ordinary skill in the art has to figure out how to do this themselves. As a result, one of ordinary skill in the art would be required to conduct an undue amount of experimentation to determine if this invention can prevent the myriad number of adverse affects that can be associated with iNO therapy.

Genetech, 108 F.3d at 1366 states that "a patent is not a hunting license. It is not a reward for search, but compensation for its successful conclusion" and "patent protection is granted in return for an enabling disclosure of an invention, not for vague intimations of general ideas that may or may not be workable." (*Genentech, Inc. v. Novo Nordisk, A/S*, 108 F.3d 1361, 1365, 42 USPQ2d 1001, 1004 (Fed. Cir. 1997)).

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1, 3, 6, 11 and 15 are rejected under 35 U.S.C. 102(b) as being anticipated by The NIH (Critical Care Therapy and Respiratory Care Section; Nitric Oxide Therapy, May 2000, 13 pages).

The NIH (Critical Care Therapy and Respiratory Care Section; Nitric Oxide Therapy, May 2000, 13 pages) discloses that administration of NO has been approved for use in the treatment of term or near term neonates and that that inhaled NO therapy is relative contraindicated for patients with severe left ventricular failure and to beware of increased left ventricular filling associated with rapid changes in pulmonary pressures (pages 2-3 of 13). A contraindication means literally contra- (against) an indication, against something that is indicated as advisable or necessary and a relative contraindication is a condition which makes a particular treatment or procedure inadvisable but does not rule it out. Therefore, in reading the guidelines set forth by the NIH one of ordinary skill in the art would practice the instant method by identifying a patient eligible for iNO treatment and then diagnosing/evaluating/screening/determining for a pre-existing left ventricular dysfunction in the patient and if present exclude the neonate from treatment to avoid/reduce the risk of the adverse events/hazards/complications but administer the treatment if the patient is not contraindicated. Claims 1 and 11 are therefore anticipated. The patient must breathe oxygen to survive and anticipates instant claims 3 and 15. All patients are 'at risk' of the events in instant claim 6, for example anyone receiving the vasodilator NO is at risk of hypotension, and therefore it is inherently anticipated.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1, 3, 5, 6, 11, 13 and 15 are rejected under 35 U.S.C. 102(b) as being anticipated by Kinsella et al. (The Lancet 1999, 354, 1061-1065).

Kinsella et al. (The Lancet 1999, 354, pp 1061-1065) disclose excluding patients (premature neonates) from inhaled nitric oxide treatment if they have fatal congenital anomalies or congenital heart disease (Abstract and page 1062, Methods). Since left ventricular dysfunction is a congenital heart disease, as acknowledged by Applicant, (see specification [0028]), and it would be pre-existing, then the methods of Kinsella et al. inherently exclude this patient population from the method. The patients also had pulmonary hypertension which would be associated with the cardiac function (Abstract). Thus, one or more adverse events are reduced in the neonates excluded from the method and instant claims 1 and 5 are anticipated. The neonate must breathe oxygen to survive and claim 3 is anticipated. Furthermore, if the patients are already excluded then any further limitations on the treatment are truly irrelevant. The intended patient population is inherently at risk of one or more adverse events and claim 6 is anticipated. Patients are inherently identified for nitric oxide inhalation treatment, diagnosed for congenital heart disease which inherently includes left ventricular dysfunction, and if the patient meets the criteria than treatment with NO is performed thereby reducing the risk of adverse events associated with the treatment. Thus instant claims 11 and 13 are anticipated. The neonate must breathe oxygen to survive and claim 15 is anticipated.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1, 3, 5, 6, 11, 13 and 15 are rejected under 35 U.S.C. 102(b) as being anticipated by Atz et al. (Seminars in Perinatology 1997, 21(5), pp 441-455).

Atz et al. disclose methods using inhaled nitric oxide in the neonate with cardiac disease (title and Abstract pages 441-453). Atz et al. disclose that: "Caution should be exercised when administering NO to patients with severe left ventricular dysfunction and pulmonary hypertension." (page 452, left column). Atz et al. continues with: "Therefore, in newborns with severe left ventricular dysfunction, predominantly left to right shunting at the foramen ovale and exclusively right to left shunting at the ductus arteriosus, ***NO should be used with extreme caution, if at all***. We and others have reported ***adverse outcomes*** in this circumstance." (page 452, left column) (Examiner added emphasis). Thus, Atz et al. fairly teaches excluding patients with left ventricular dysfunction from inhaled NO treatment because the Examiner interprets "if at all" to mean no treatment and hence exclusion from treatment and consequently any adverse events are reduced. The left ventricular dysfunction is inherently pre-existing and therefore instant claim 1 is anticipated. The neonate has to breathe oxygen to survive thus reading on instant claim 3. Atz et al. teach neonates with pulmonary hypertension (Abstract and page 442, left column to right column) which reads on instant claim 5. The patient population is inherently at risk of the events of instant claim 6. The methods disclosed by Atz et al. are interpreted to

mean identifying a patient eligible for NO treatment; diagnosing if the patient has left ventricular dysfunction; excluding that patient with left ventricular dysfunction from treatment with NO but treating the patient with NO for other conditions discussed by Atz et al. with inhalation of NO thereby reducing the risk of adverse events associated with the medical treatment. Therefore, instant claims 11, 13, and 15 are anticipated for the reasons discussed above.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* **v.** *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

Claims 1-19 are rejected under 35 U.S.C. 103(a) as being unpatentable over Atz et al. (Seminars in Perinatology 1997, 21(5), pp 441-455) and Kinsella et al. (The Lancet 1999, 354, 1061-1065) and Bolooki (Clinical Application of the Intra-Aortic Balloon Pump 1998, 3rd Ed. Pp

252-253) and Loh et al. (Circulation 1994, 90, 2780-2785) and The NIH (Critical Care Therapy and Respiratory Care Section; Nitric Oxide Therapy, May 2000, 13 pages).

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Applicant claims:

> 1. A method of reducing one or more adverse events or serious adverse events in an intended patient population comprising neonates or near-term neonates in need of being treated with inhalation of nitric oxide comprising excluding from such treatment anyone in the intended patient population having pre-existing left ventricular dysfunction.

Determination of the scope and content of the prior art

(MPEP 2141.01)

Atz et al. teach methods using inhaled nitric oxide in the neonate with cardiac disease (title and Abstract). Atz et al. teach that: "Caution should be exercised when administering NO to patients with severe left ventricular dysfunction and pulmonary hypertension." (page 452, left

column). Atz et al. continues with: "Therefore, in newborns with severe left ventricular dysfunction, predominantly left to right shunting at the foramen ovale and exclusively right to left shunting at the ductus arteriosus, ***NO should be used with extreme caution, if at all***. We and others have reported ***adverse outcomes*** in this circumstance." (page 452, left column) (Examiner added emphasis). Thus, Atz et al. fairly teaches excluding patients with left ventricular dysfunction from inhaled NO treatment because the Examiner interprets "if at all" to mean no treatment and hence exclusion from treatment. The left ventricular dysfunction is intrinsically pre-existing. The methods disclosed by Atz et al. are interpreted to mean identifying a patient eligible for NO treatment; diagnosing if the patient has left ventricular dysfunction; excluding that patient with left ventricular dysfunction from treatment with NO but treating the patient with NO for other conditions discussed by Atz et al. with inhalation of NO thereby reducing the risk of adverse events associated with the medical treatment. Atz et al. teach neonates with pulmonary hypertension (Abstract and page 442, left column to right column).

Bolooki teaches using intra-aortic balloon pump as well as nitroglycerin and calcium channel blockers in the treatment of left ventricular dysfunction (pages 252-253).

The NIH (Critical Care Therapy and Respiratory Care Section; Nitric Oxide Therapy, May 2000, 13 pages) establishes that inhaled NO therapy is relative contraindicated for patients with severe left ventricular failure and to beware of increased left ventricular filling associated with rapid changes in pulmonary pressures (pages 2-3 of 13). Thus, it is a medical mainstream concept that inhaled NO therapy is relative contraindicated for patients with left ventricular dysfunction.

The references of Kinsella et al. and Loh et al. are discussed in detail above and those discussions are hereby incorporated by reference.

Ascertainment of the difference between the prior art and the claims

(MPEP 2141.02)

1. The difference between the instant application and Atz et al. is that Atz et al. do not expressly teach a method of excluding patients from treatment with a pulmonary capillary wedge pressure greater than 20 mm Hg or using a ventilator for the treatment or idiopathic pulmonary arterial hypertension/congenital heart disease/cardiomyopathy or catheterization characterized by different means. This deficiency in Atz et al. is cured by the teachings of Kinsella et al., Loh et al., The NIH and common sense.

2. The difference between the instant application and Atz et al. is that Atz et al. do not expressly teach: reducing the left ventricular afterload with nitroglycerin, calcium channel blocker or intra-aortic balloon pump such that pulmonary edema is reduced/minimized. This deficiency in Atz et al. is cured by the teachings of Bolooki.

Finding of prima facie obviousness

Rational and Motivation (MPEP 2142-2143)

1. It would have been obvious to one of ordinary skill in the art at the time the claimed invention was made to perform the method of Atz et al. and exclude patients with left ventricular dysfunction wherein the intended patient population is excluded that has a pulmonary capillary wedge pressure greater than 20 mm Hg or using a ventilator for the treatment or idiopathic

pulmonary arterial hypertension/congenital heart disease/cardiomyopathy or catheterization characterized by different means, as suggested by Loh et al., the NIH and Kinsella et al., and produce the instant invention.

One of ordinary skill in the art would have been motivated to do this because Atz et al. clearly teach using extreme caution or not using NO at all in the treatment of patients with left ventricular dysfunction and the art of Kinsella et al. establishes excluding certain patients from treatment. Furthermore, the NIH teaches that inhaled NO therapy is relative contraindicated for patients with left ventricular dysfunction. ***In other words, the art is already well aware and informed that inhaled NO is contraindicated for patients with left ventricular dysfunction, and consequently it is not inventive to exclude that patient population from treatment when the art already suggests it!*** Thus it is no stretch of the imagination to exclude patients with left ventricular dysfunction with or without the myriad number of other conditions characterized by various medical parameters claimed by Applicant from inhaled nitric oxide therapy in order to avoid adverse outcomes as taught by Atz et al. which intrinsically include all the adverse events recited by Applicant. The ordinary artisan would err on the side of caution for the benefit of the patient. Such patients intrinsically have a pulmonary capillary wedge pressure of greater than 20 mm Hg. In other words, the teachings of Atz et al. include the patients with left ventricular dysfunction intrinsically that have a pulmonary capillary wedge pressure of greater than 20 mm Hg. Inhaled NO increased the wedge pressure as taught by Loh et al. (see entire document) Furthermore, it is merely a design choice by the ordinary artisan to select a ventilator to administer the gas. This is just common sense.

Furthermore, with respect to instant claims 3 and 4, if the patients are already excluded from treatment then any further limitations on the treatment are truly irrelevant.

2. It would have been obvious to one of ordinary skill in the art at the time the claimed invention was made to reducing the left ventricular afterload with nitroglycerin, calcium channel blocker or intra-aortic balloon pump such that pulmonary edema is reduced/minimized**,** as suggested by Bolooki, and produce the instant invention.

One of ordinary skill in the art would have been motivated to do this because administration of nitroglycerin, calcium channel blocker or intra-aortic balloon pump to treat left ventricular dysfunction is a common technique in the art as taught by Bolooki and intrinsically reduces the left ventricular afterload and reduces pulmonary edema.

In light of the forgoing discussion, the Examiner concludes that the subject matter defined by the instant claims would have been obvious within the meaning of 35 USC 103(a).

From the teachings of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible

harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

1. Claims 1-19 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-30 of copending Application No. 12/494598. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant subject matter embraces or is embraced by the subject matter of the copending subject matter. Both applications are drawn to methods of reducing one or more

adverse events in a patient population by excluding from treatment anyone with pre-existing left ventricular dysfunction.

The copending application does not expressly teach near-term neonates or neonates.

However the copending broadly teaches an intended patient population which would include neonates and near-term neonates.

Therefore one of ordinary skill in the art would have recognized the obvious variation of the instant application over the copending application.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

2. Claims 1-19 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-19 of copending Application No. 12/820980. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant subject matter embraces or is embraced by the subject matter of the copending subject matter. Both applications are drawn to methods of reducing one or more adverse events in a patient population by excluding from treatment anyone with pre-existing left ventricular dysfunction.

The copending application does not expressly teach near-term neonates or neonates.

However the copending broadly teaches children patient population which would include neonates and near-term neonates as clearly neonates are children. The instant specification defines 'children' as being 4 weeks old [0023] which would be newborn and hence neonatal.

Therefore one of ordinary skill in the art would have recognized the obvious variation of the instant application over the copending application.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

3. Claims 1-19 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of copending Application No. 12/821020. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant subject matter embraces or is embraced by the subject matter of the copending subject matter. Both applications are drawn to methods of reducing one or more adverse events in a patient population by excluding from treatment anyone with pre-existing left ventricular dysfunction.

The copending application does not expressly teach the intended population as having one or more conditions.

However the copending application is drawn to the same patient population which intrinsically has one or more conditions as instantly claimed.

Therefore one of ordinary skill in the art would have recognized the obvious variation of the instant application over the copending application.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

4. Claims 1-19 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of copending Application No. 12/821041. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant subject matter embraces or is embraced by the subject matter of the copending subject matter. Both applications are drawn to methods of reducing one or more adverse events in a patient population by excluding from treatment anyone with pre-existing left ventricular dysfunction.

The copending application does not expressly teach the intended population as having one or more conditions.

However the copending application is drawn to the same patient population which intrinsically has one or more conditions as instantly claimed.

Therefore one of ordinary skill in the art would have recognized the obvious variation of the instant application over the copending application.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Conclusion

No claims are allowed. No allowable subject can be discerned.

Applicant is reminded that for any amendments to the claims (including any new claim) that is not encompassed by the preexamination search and accelerated examination support documents previously filed, applicant is required to provide updated preexamination search and

accelerated examination support documents that encompass the amended or new claims at the time of filing the amendment. Failure to provide such updated preexamination search and accelerated examination support documents at the time of filing the amendment will cause the amendment to be treated as not fully responsive and not to be entered. See MPEP § 708.02(a) subsection VIII.D. for more information.

If the reply is not fully responsive, the final disposition of the application may occur later than twelve months from the filing of the application.

Any reply or other papers must be filed electronically via EFS-Web so that the papers will be expeditiously processed and considered. If the papers are not filed electronically via EFS-Web, the final disposition of the application may occur later than twelve months from the filing of the application.

Any reply to this communication filed via EFS-Web must include a document that is filed using the document description of "Accelerated Exam - Transmittal amendment/reply." Applicant is reminded to use proper indexing for documents to avoid any delay in processing of follow on papers. Currently document indexing is not automated in EFS-Web and applicant must select a particular document description for each attached file. An incorrect document description for a particular file may potentially delay processing of the application. A complete listing of all document codes currently supported in EFS-Web is available at http://www.uspto.gov/ebc/portal/efs/efsweb_document_descriptions.xls.

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payment of fees via EFS-Web are available at http://www.uspto.gov/ebc/portal/efs/quick-start.pdf.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ernst V. Arnold whose telephone number is 571-272-8509. The examiner can normally be reached on M-F (7:15 am-4:45 pm).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Johann Richter can be reached on 571-272-0646. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

/Ernst V Arnold/
Primary Examiner, Art Unit 1616

Notice of References Cited	Application/Control No. 12/820,866	Applicant(s)/Patent Under Reexamination BALDASSARRE ET AL.	
	Examiner ERNST V. ARNOLD	Art Unit 1616	Page 1 of 2

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
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*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	The American Illustrated Medical Dictionary (Dorland, 1914, 7th Ed, page 113)
	V	Beghetti et al. (Journal of Pediatrics, 1997, page 844).
	W	Macrae et al. (Intensive Care Med 2004, 30, pp 372-380)
	X	Atz et al. (Seminars in Perinatology 1997, 21(5), pp 441-455)

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Kinsella et al. (The Lancet 1999, 354, pp 1061-1065)
	V	The NIH (Critical Care Therapy and Respiratory Care Section; Nitric Oxide Therapy, May 2000, 13 pages)
	W	Bolooki (Clinical Application of the Intra-Aortic Balloon Pump 1998, 3rd Ed. Pp 252-253)
	X	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99**)		
	Application Number	
	Filing Date	
	First Named Inventor	James S. Baldassarre
	Art Unit	
	Examiner Name	
	Attorney Docket Number	I001-0002USC1

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	2	6063407		2000-05-16	Zapol, ; et al.	
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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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	2	20090018136	A1	2009-01-15	Oppenheimer; Daniel I.; et al.	
	3	20090029371	A1	2009-01-29	Elliott; C. Gregory	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /E.A./

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	First Named Inventor	James S. Baldassarre
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	Examiner Name	
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	1	EP1682672;(A1)			2006-07-26	COUNCIL SCIENT IND RES [IN]+ (COUNCIL O		☐
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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	12820866 - GAU: 1616
	Filing Date	
	First Named Inventor	James S. Baldassarre
	Art Unit	
	Examiner Name	
	Attorney Docket Number	I001-0002USC1

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /E.A./

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	12820866 - GAU: 1616
	Filing Date	
	First Named Inventor	James S. Baldassarre
	Art Unit	
	Examiner Name	
	Attorney Docket Number	I001-0002USC1

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	Application Number	
	Filing Date	
	First Named Inventor	James S. Baldassarre
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	Examiner Name	
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	Examiner Name	
	Attorney Docket Number	I001-0002USC1

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12820866	BALDASSARRE ET AL.
	Examiner	Art Unit
	ERNST V ARNOLD	1616

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
inventor name EAST/PALM	8/10/10	eva
EAST 424/718 text limited all databases	8/10/10	eva
google	8/10/10	eva

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
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L2	2	"6063407".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/11 07:33
L3	2	"6601580".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/11 07:33
L4	2	"7557087".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/11 07:33
L5	151	((ventricular adj dysfunction) and (iNO or (inhaled with (nitric adj oxide))))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/11 07:45
L6	146	l5 and @ad<"20090630"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/11 07:45
L7	2	l6 and ((ventricular adj dysfunction) and (iNO or (inhaled with (nitric adj oxide)))).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/11 07:46
L8	0	l6 and ((ventricular adj dysfunction) and (nitric adj oxide)).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/11 07:47

L9	75	l6 and ((ventricular adj dysfunction) and (nitric adj oxide) and (inhale or inhaled or inhalation))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/11 07:47
L10	17	l6 and ((ventricular adj dysfunction) and (nitric adj oxide).clm. and (inhale or inhaled or inhalation))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/11 07:48

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	1750	((ventricular with (dysfunction or afterload)) and (nitroglycerin or (calcium with blocker) or (aortic with balloon)))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:12
S2	1704	S1 and @ad<"20090630"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:13
S3	142	S2 and ((inhaled with (nitric adj oxide)) or iNO)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:13
S4	9	S3 and ((ventricular with (dysfunction or afterload)) and (nitroglycerin or (calcium with blocker) or (aortic with balloon))). clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:14
S5	9	S3 and ((ventricular with (dysfunction or afterload)) and (nitroglycerin or (calcium with blocker) or (intraaortic with balloon))). clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:19
S6	596	S1 and (exclude or exclusion)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:19
S7	77	S1 and ((exclude or exclusion) with (patient or subject or child or mammal))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:20
S8	76	S7 and @ad<"20090630"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:20

S9	13	S8 and ((iNO or NO or (nitric adj oxide)) with (inhaled or inhale or breathe or inhalation))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:23
S10	57	S1 and ((iNO or NO or (nitric adj oxide)) with (inhaled or inhale or breathe or inhalation))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:24
S11	56	S10 and @ad<"20090630"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:25
S12	31	S11 and (exclude or exclusion or excluded)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:25
S13	1	((ventricular with (dysfunction or afterload)) and (nitroglycerin and ((calcium adj channel) with blocker) and (intraaortic with balloon)))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:36
S14	3	((ventricular with (dysfunction or afterload)) and (nitroglycerin and (calcium with blocker) and (intraaortic with balloon)))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:37
S15	0	baldassarre.in. and (ventricular with dysfunction)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:41
S16	0	rosskamp.in. and (ventricular with dysfunction)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:41
S17	6	424/718.ccls. and (ventricular with dysfunction)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:41

S18	6	S17 and @ad<"20090630"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2010/08/09 17:42

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE (USPTO)	
Application Serial Number	12/820,866
Confirmation Number	2913
Filing Date	June 22, 2010
Title of Application	Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension
First Named Inventor	James S. Baldassarre
Assignee	Ikaria, Inc.
Group Art Unit	1616
Examiner	Arnold, Ernst V.
Attorney Docket Number	I001-0002USC1

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Alexandria, VA 22313-1450

REPLY AMENDMENT (37 CFR § 1.111)
ACCELERATED EXAM--TRANSMITTAL AMENDMENT/REPLY

This communication is responsive to the **Non-Final Office Action Summary for Applications Under Accelerated Examination** on the merits dated September 23, 2010, setting a 30 day or 1 month time period for response pursuant to grant of the Petition to Accelerate Examination.

Included herein are Declarations under 35 USC § 1.132 by the inventor, Dr. James S. Baldassarre, M.D. and by Jeffrey R. Smith, both of whom are experts in the medical field of the instant invention.

Applicants respectfully request entry of this Reply Amendment, reconsideration of the pending rejections, and allowance of the application.

A listing of the claims and amendments thereof is shown starting at page 2.

Remarks to the pending Office Action begin at page 6.

Listing of Claims

1. (Presently Amended) A method of reducing the risk of one or more adverse events or serious adverse events in an intended patient population comprising neonates or near-term neonates in need of being treated with inhalation of nitric oxide comprising excluding from such treatment anyone in the intended patient population having pre-existing left ventricular dysfunction.

2. (Presently Amended) ~~The method of claim 1,~~ A method of reducing the risk of one or more adverse events or serious adverse events in an intended patient population comprising neonates or near-term neonates in need of being treated with inhalation of nitric oxide comprising excluding from such treatment anyone in the intended patient population having pre-existing left ventricular dysfunction, wherein anyone in the intended patient population further has a pulmonary capillary wedge pressure greater than 20 mm Hg.

3. (Presently Amended) The method of claim 2 ~~1~~, wherein the treatment further comprises inhalation of oxygen.

4. (Presently Amended) The method of claim 2 ~~1~~, wherein the treatment is delivered using a ventilator.

5. (Presently Amended) The method of claim 2 ~~1~~, wherein anyone in the intended patient population having pre-existing left ventricular dysfunction also having one or more conditions selected from diastolic dysfunction, hypertensive cardiomyopathy, systolic dysfunction, ischemic cardiomyopathy, viral cardiomyopathy, idiopathic cardiomyopathy, autoimmune disease related cardiomyopathy, drug-related cardiomyopathy, toxin-related cardiomyopathy, structural heart disease, valvular heart disease, congenital heart disease, idiopathic pulmonary arterial hypertension and pulmonary hypertension cardiomyopathy~~, and associations thereof~~.

6. (Presently Amended) The method of claim 2 ~~1~~, wherein the intended patient population are at risk of one or more adverse events or serious adverse events selected from pulmonary edema, hypotension, cardiac arrest, electrocardiogram changes, hypoxemia, hypoxia and bradycardia~~, and, associations thereof~~.

7. (Presently Amended) The method of claim 2 ~~1~~, further comprising reducing left ventricular afterload to minimize or reduce the risk of the occurrence of an adverse event or serious adverse event being pulmonary edema.

8. (Original) The method of claim 7, wherein the left ventricular afterload is minimized or reduced by administering a pharmaceutical dosage form comprising nitroglycerin or calcium channel blocker.

9. (Original) The method of claim 7, wherein the left ventricular afterload is minimized or reduced using an intra-aortic balloon pump.

10. (Presently Amended) The method of claim 2 ~~1~~, wherein the intended patient population in need of being treated with the inhalation of nitric oxide has one or more of idiopathic pulmonary arterial hypertension characterized by PAPm > 25 mm Hg at rest, PCWP ≤ 15 mm Hg, and, a PVRI > 3 u·m^2; congenital heart disease with pulmonary hypertension repaired and unrepaired characterized by PAPm > 25 mm Hg at rest and PVRI > 3 u·m^2; cardiomyopathy characterized by PAPm > 25 mm Hg at rest and PVRI > 3 u·m^2 ; or, the patient is scheduled to undergo right heart catheterization to assess pulmonary vasoreactivity by acute pulmonary vasodilation testing.

11. (Presently Amended) A method of reducing the risk of ~~or preventing~~ the occurrence, in a patient being a neonate or near-term neonate, of one or more adverse events or serious adverse events associated with a medical treatment comprising inhalation of nitric oxide, said method comprising:

a. identifying a patient eligible for inhalation of nitric oxide treatment;

b. determining if said patient has pre-existing left ventricular dysfunction evidenced by an elevated pulmonary capillary wedge pressure; and,

c. administering said medical treatment if said patient does not have pre-existing left ventricular dysfunction, wherein said patient is excluded from being administered said medical treatment if said patient has pre-existing left ventricular dysfunction;

thereby reducing the risk of ~~or preventing~~ the occurrence of the adverse event or serious adverse event associated with said medical treatment.

12. (Original) The method of claim 11, wherein said patient further exhibits a pulmonary capillary wedge pressure greater than 20 mm Hg.

13. (Presently Amended) The method of claim 11, wherein the patient who has pre-existing left ventricular dysfunction has one or more conditions selected from diastolic dysfunction, hypertensive cardiomyopathy, systolic dysfunction, ischemic cardiomyopathy, viral cardiomyopathy, idiopathic cardiomyopathy, autoimmune disease related cardiomyopathy, side effects due to drug-related cardiomyopathy, side effects due to toxin-related cardiomyopathy, structural heart disease, valvular heart disease, congenital heart disease, idiopathic pulmonary arterial hypertension, pulmonary hypertension and cardiomyopathy~~, and associations thereof~~.

14. (Presently Amended) The method of claim 12, wherein the patient who has pre-existing left ventricular dysfunction has one or more conditions selected from diastolic dysfunction, hypertensive cardiomyopathy, systolic dysfunction, ischemic cardiomyopathy, viral cardiomyopathy, idiopathic cardiomyopathy, autoimmune disease related cardiomyopathy, side effects due to drug-related cardiomyopathy, side effects due to toxin-related cardiomyopathy, structural heart disease, valvular heart disease, congenital heart disease, idiopathic pulmonary arterial hypertension, pulmonary hypertension and cardiomyopathy~~, or associations thereof~~.

15. (Original) The method of claim 11, wherein the medical treatment further comprises inhalation of oxygen.

16. (Original) The method of claim 11, wherein the treatment is delivered using a ventilator.

17. (Original) The method of claim 11, further comprising reducing left ventricular afterload to minimize or reduce the risk of the occurrence of an adverse event or serious adverse event being pulmonary edema.

18. (Original) The method of claim 17, wherein the left ventricular afterload is minimized or reduced by administering a pharmaceutical dosage form comprising nitroglycerin or calcium channel blocker.

19. (Original) The method of claim 17, wherein the left ventricular afterload is minimized or reduced using an intra-aortic balloon pump.

REMARKS

Claims 1-19 are pending in the application. The application contains 3 independent claims (i.e., claims 1, 2 and 11), 19 total claims, and no multiple dependent claims. As such, the application complies with the 3/20 claims limitation for accelerated applications.

Claims 1-7, 10, 11, 13 and 14 have been amended to more particularly point out and distinctly claim the subject matter applicant regards as the invention. The claims have been amended without prejudice to said subject matter being claimed in a continuing application. Support for the amendments is found in the application as filed. No new matter has been added. For example, the recitation added to claim 11 is found at [0029] of the application as originally filed.

No new claims have been added.

At page 1 of the Office Action, Applicant acknowledges at ¶¶ 3 and 5 of that claims 1-19 stand pending and rejected. Applicant further acknowledges Attachments 1 and 3 of the Office Action indicating entry of the PTO-892 and IDS, respectively.

At page 2 of the Office Action, Applicant further acknowledges denial of Applicant's request for an interview with the examiner. Applicant notes, however, that an interview was conducted with Examiner Arnold in Applicant's related continuation applications.[1] Applicant further notes that an Office Action was entered by Examiner Arnold in a co-pending application claiming substantially similar subject matter.[2]

Rejections Under 35 USC § 112

At page 2 of the Office Action, claims 1-19 stand rejected under 35 USC § 112, 2nd Paragraph, on the grounds that the recitation "serious adverse event" is indefinite

[1] See, Interview Summary dated September 7, 2010 in Application Serial 12/821,041 and Interview Summary dated September 9, 2010 in Application Serial No. 12/821,020. See also, Applicant's Examiner Interview agendas dated August 30, 2010 in each case respectively. Applicant thanks Examiner Arnold for the courtesies extended during the telephonic interview conducted on September 1, 2010, in 12/821,041. Applicant further notes that Examiner Arnold is the examiner of record in Applicant's 3 co-pending continuation applications: Serial Nos. 12/820,980; 12/821,020; and, 12/821,041 as well as the parent application Serial No. 12/494,598, which has been expressly abandoned.
[2] See, US Serial No. 12/820,980 dated August 17, 2010.

and that the recitations "serious adverse event" and "adverse event" overlap and/or are redundant. Applicant respectfully traverses the rejection and assertions thereof because one of ordinary skill in the medical arts would clearly recognize and understand what those terms mean, the differences thereof, that they are not redundant, and, the non-overlapping boundaries thereof.

The definition of "adverse event" is clearly set forth in the specification as originally filed.[3] The definition of "serious adverse event" is also clearly set forth in the application as originally filed.[4] As set forth in the attached Declaration by Jeffrey R. Smith, Esq., an expert in the field of pediatric intensive care arts, adverse events and serious adverse events have well understood and defined meanings to those of ordinary skill and to the U.S. FDA.[5]

The FDA, in Federal regulations 21 CFR Part 312, defines adverse events as any untoward medical occurrence that may present itself during treatment or administration with a pharmaceutical product, and which may or may not have a causal relationship with the treatment. In the guideline entitled “Clinical Safety Data Management: Definitions and Standards for Expedited Reporting”, the Agency further clarifies and defines serious adverse events stemming from a drug study as any untoward medical occurrence that at any dose results in death; is life-threatening; requires inpatient hospitalization or prolongation of existing hospitalization; creates persistent or significant disability/incapacity, or a congenital anomaly/birth defects. Reconsideration and withdrawal of the rejection is respectfully requested.

At page 3 of the Office Action, claims 5, 6, 13 and 14 stand rejected under 35 USC § 112, 2nd Paragraph, as being indefinite on the ground that the recitation "associations thereof" is unclear. Applicant respectfully traverses the rejection and assertion thereof. However, to facilitate allowance of the application, the recitation has been deleted. Reconsideration and withdrawal of the rejection is respectfully requested.

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[3] See ¶[0026].
[4] See ¶[0028].
[5] See, Smith Declaration at ¶¶11-15.

At pages 3-8 of the Office Action, claims 11-19 stand rejected under 35 USC § 112, 1st Paragraph, on the ground that the claims are nonenabled for "preventing" adverse events and serious adverse advents. Applicant respectfully traverses the rejection and the assertions thereof. However, to facilitate allowance of the application, the recitation has been deleted. Reconsideration and withdrawal of the rejection is respectfully requested.

Rejections Under 35 USC § 102(b)

At pages 8-9 of the Office Action, claims 1, 3, 6, 11 and 15 stand rejected under 35 USC § 102(b) as being anticipated by *NIH Clinical Center, Department Policy and Procedure Manual for the Critical Care Therapy and Respiratory Care Section; Nitric Oxide Therapy, 2000*. (Hereinafter "NIH manual"). Applicant respectfully traverses the rejection and the assertions thereof.

As set forth in the Smith Declaration[6], the NIH manual sets forth internal policy and procedure for respiratory therapists working in the Medical Intensive Care Unit of the Magnuson Clinical Center--a NIH hospital. Such therapists are responsible for administering inhaled nitric oxide (iNO).

Notably, the Magnuson Clinical Center does not have a neonatal intensive care unit,[7] and adult patients are admitted to the 12 bed MICU, which is inapplicable to neonates. In particular, at § 3.1.2 of the NIH manual entitled "Clinical Support of Inpatients," it states that "NO may be used to support critically ill patients who present to the MICU with severe pulmonary hypertension due to acute respiratory failure." There is also no mention of elevated PCWP or PCWP>20 mmHg in the NIH manual.

At § 5.2.3 of the NIH manual, it discloses that iNO has a "Relative contraindication" for "Severe left ventricular failure." However, as set forth *supra*, the NIH manual does not apply to neonates or near-term neonates because the hospital does not have a neonatal ICU. As such, the NIH manual only applies to adults. And, as set forth in the Smith Declaration, neonates or near-term neonates cannot be treated as

[6] See ¶47.
[7] See, http://www.cc.nih.gov/ccmd/clinical_services.html. See also, Exhibit B and fn. 27 of Smith Declaration.

adults in terms of iNO treatment.[8] Moreover, a relative contraindication (i.e., cautionary) is not an exclusion as required by the recitation "excluding" in the instantly claimed invention.[9] Moreover, the NIH manual presents no data to support the relative contraindication for iNO for adult inpatients diagnosed with severe left ventricular failure. The only "Absolute contraindications" disclosed by the NIH manual are for "patients with congenital or acquired methemglobinemia reductase deficiency."[10]

To anticipate a claim, a single prior art reference must be enabled and teach each and every element of the claimed invention.[11] In this case, the NIH manual fails to anticipate the claimed invention because, *inter alia*, it is nonenabling and does not disclose excluding neonates or near-term neonates from receiving iNO where such neonates or near-term neonates have pre-existing left ventricular dysfunction ("LVD"), a PCWP>20 mmHg, or, an elevated PCWP. As set forth in the Smith Declaration, a subject characterized by a PCWP>20 mm Hg is but one means to diagnose pre-existing LVD.[12] The NIH manual also fails to explicitly mention reducing the risk of adverse events or serious adverse events.

The Office Action argues that the NIH article inherently anticipates the claimed invention. With all due respect, the doctrine of inherency is used to assert that a prior art reference, without stating such, possesses a claimed property, characteristic or the like.[13] For example, a mixture of NaCl and water inherently contains Na^+ ions and Cl^- ions in the aqueous solution. Absent language suggesting it, one would not add NaCl to water unless the reference teaches the act of adding NaCl to water. In this case, the NIH article does not inherently disclose excluding neonates or near-term neonates with pre-existing LVD (such as those characterized by an elevated PCWP or a PCWP>20 mmHg) because it does not state such an affirmative act.

Reconsideration and withdrawal of the anticipation rejection over the NIH manual is respectfully requested.

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[8] See ¶¶42-46.
[9] See, Smith Declaration at ¶¶16-28.
[10] §§ 5.1 and 5.1.1.
[11] MPEP 2131.
[12] See, ¶¶29-35.
[13] See, MPEP 2112. See also, MPEP 2112.02.

At pages 9-10 of the Office Action, claims 1, 3, 5, 6, 11, 13 and 15 stand rejected under 35 USC § 102(b) as being anticipated by *Kinsella, et al, The Lancet,* (1999) ("Kinsella").

As set forth in the Smith Declaration[14], Kinsella involves a double blind study that evaluated 80 premature infants with severe hypoxic respiratory failure.[15] The exclusion criteria was "fatal congenital anomolies or gengenital heart disease (except atrial and ventricular septal defects."[16] The patients were randomized into two groups--iNO administered to one group but not the other.[17] The rate and severity of intracranial hemorrhage, pulmonary hemorrhage, duration of ventilation, and, chronic lung disease were also studied in the two groups.[18] Kinsella noted the potential adverse effects of iNO on platelet adhesion.[19] Kinsella also noted the attendant risks of intracranial hemorrhage and the severe consequences of prematurity.[20]

Kinsella concluded that low dose iNO improved oxygenation and decreased the need for mechanical ventilation as well as lowered the frequency of chronic lung disease but did not improve survival in severely hypoxic neonates.[21] However, Kinsella is silent concerning neonates or near-term neonates with pre-existing LVD being excluded from treatment with iNO. Kinsella is also silent respecting the instantly claimed elevated PCWP and PCWP>20 mmHg as exclusionary criteria.

Again, to anticipate a claim, a single prior art reference must be enabled and teach each and every element of the claimed invention.[22] In this case, Kinsella fails to anticipate the claimed invention because, *inter alia*, it is does not disclose excluding neonates or near-term neonates from receiving iNO where such neonates or near-term neonates have pre-existing left ventricular dysfunction ("LVD"), a PCWP>20 mmHg, or,

[14] See ¶50.
[15] See, page 1061, first dolumn.
[16] Page 1062, first column.
[17] Id.
[18] Page 1063, first column.
[19] Page 1064, first column.
[20] Id.
[21] Id.
[22] MPEP 2131.

an elevated PCWP. Kinsella also fails to explicitly mention reducing the risk of adverse events or serious adverse events.

The Office Action again argues that the affirmatively claimed method steps are inherently disclosed in Kinsella. Applicant respectfully traverses this inherency assertion for the reasons set forth *supra* respecting the NIH anticipation rejection.

Reconsideration and withdrawal of the Kinsella anticipation rejection is respectfully requested.

At pages 11-12 of the Office Action, claims 1, 3, 5, 6, 11, 13 and 15 stand rejected under 35 USC § 102(b) as being anticipated by *Atz, et al., Seminars in Perinatology, 1997* ("Atz"). Applicant respectfully traverses the rejection and the assertions thereof.

Atz is a review of data collected from the treatment of 400 patients (37% newborns) with iNO.[23] Nearly two-thirds of those patients exhibited pulmonary hypertension associated with congenital heart disease.[24] Patients underwent early surgical repair of their congenital heart defects at Boston's Children's Hospital.[25]

Atz discloses the use of iNO in newborns with severe LVD and pulmonary hypertension.[26] Atz also points out that administration of iNO to newborns should be with "extreme caution, if at all."[27] Atz also discloses that the newborn patients had a specific set of anatomic and hemodynamic characteristics, whereby the systemic circulation may depend in part on the ability of the right ventricle to sustain cardiac output via a right-to-left shunt across the patient's ductus arteriosus.[28]

Atz cites other studies reporting adverse outcomes under such circumstances.[29] Hence, even considering the referenced articles commenting that iNO should be used

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[23] See, Abstract.
[24] See, page 443, first column.
[25] Id.
[26] Page 452, first column.
[27] Id.
[28] See, Abstract.
[29] Wessel, et al., Improved Oxygenation in a Randomized Trial of Inhaled Nitric Oxide for Persistent Pulmonary Hypertension of the Newborn, Pediatrics, Vol. 100, Number 5, 1997. In this study, the authors randomized 49

"with extreme caution, if at all," nowhere does the article suggest that patients with LVD should be **excluded** from therapeutic treatment with iNO due to a risk of pulmonary edema. As argued *supra*, the recitation "extreme caution" is not the same as the instant claim recitation "excluding." Moreover, although the authors add the recitation "if at all," this qualification is at odds with the data provided in the study, and, the report clearly states, at p. 452, second column, that "no randomized placebo-controlled" studies had been performed, which renders the "extreme caution" opinion nonenabled.

As to claims 3, 5 and 6, Atz fails to disclose, *inter alia*, the instant claim recitation "wherein anyone in the intended patient population further has a pulmonary capillary wedge pressure greater than 20 mm Hg."

As to claims 11, 13 and 15, Atz fails to disclose, *inter alia*, "determining if said patient has pre-existing left ventricular dysfunction evidenced by an elevated pulmonary capillary wedge pressure." As set forth in the Smith Declaration, a subject characterized by a PCWP>20 mm Hg is but one means to diagnose pre-existing LVD.[30]

To anticipate a claim, a single prior art reference must be enabled and teach each and every element of the claimed invention.[31] In this case, Atz fails to anticipate the claimed invention because, *inter alia*, it is nonenabling and does not disclose excluding neonates or near-term neonates with elevated PCWP, PCWP>20 mmHg, or, pre-existing LVD from receiving iNO. Moreover, Atz fails to disclose reducing the risk of adverse event or serious adverse events by screening and excluding neonates or near-term neonates having elevated PCWP, PCWP>20 mmHg or pre-existing LVD fom receiving iNO.

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mechanically ventilated newborns with severe persistent pulmonary hypertension. Patients with gestational age of less than 34 weeks or with congenital heart disease or diaphragmatic hernia were excluded from the study. However, there was no mention of exclusion of newborns diagnosed with pre-existing LVD. One patient in this study later identified with "poor left ventricular function and a right ventricular dependent circulation with echocardiographic evidence of a small left atrium and left atrial hypertension with continuous right to left ductal flow," died during the study. This was after iNO was administered and systemic hypotension resulted, which led to initiation of ECMO and the patient died from an intracranial hemorrhage. This article is silent with respect to neonates and/or near-term-neonates being excluded from being administered iNO. See also, Smith Declaration at ¶48 and fn. 29.

[30] See, ¶¶29-35.

[31] See, MPEP 2112. See also, MPEP 2112.02.

The Office Action argues that Atz inherently anticipates the claimed invention. As argued *supra*, the doctrine of inherency is used to assert that a prior art reference, without stating such, possesses a property, characteristic or the like. In this case, Atz does not inherently disclose excluding neonates or near-term neonates with pre-existing LVD (particularly those having a PCWP>20mm Hg) because it does not state such an affirmative act.

Reconsideration and withdrawal of the anticipation rejection over Atz is respectfully requested.

Rejections Under 35 USC § 103(a)

At pages 12-17 of the Office Action, claims 1-19 stand rejected under 35 USC § 103(a) over **five** different references. In addition to the NIH manual, Kinsella and Atz applied *supra*, the omnibus obviousness rejection includes two additional references: *Loh, E., et al., Circulation* (1994) ("Loh") and *Bolooki, Textbook, Clinical Application of the Intra-Aortic Balloon Pump*, (1998) ("Bolooki"). Applicant respectfully traverses the rejection and the assertions thereof.

Although each one of these references will be addressed in detail below, Applicant wishes to reiterate the facts discussed during the interviews conducted in two co-pending continuation applications[32] surrounding the INOT22 trial and the present invention.

As detailed in the Declaration of Dr. James Baldassarre[33], INO Therapeutics LLC (INO) sponsored a clinical trial (the INOT22 trial) to study the use of inhaled nitric oxide in pulmonary reactivity. The INOT22 study was established and designed by INO and a Steering Committee comprising internationally recognized experts in the field of pediatric heart and lung disease, whose members would assist INO to develop the

[32] See, *supra*.
[33] See ¶¶4-6

INOT22 protocol, monitor the progress of the trial, and provide recommendations to INO on changes in the procedures and conduct of the trial.[34]

The exclusion criteria of the original NOT22 protocol **did not exclude** from the trial patients with pre-existing LVD.[35] The original INOT22 protocol and investigation plan was further reviewed, and approved by the Institutional Review Board (IRB) and/or Independent Ethics Committee (IEC) at each of the participating study institutions, including review by the principal investigator within each study institution.[36]

At no time did any member of the Steering Committee, nor any member of an IRB, IEC, or individual principal investigator, appreciate, recognize or otherwise suggest that the exclusion criteria be amended to exclude study subjects with pre-existing left ventricular dysfunction (LVD), due to an anticipated or predicted risk of adverse events or serious adverse events arising from the use of iNO in patients with pre-existing LVD, and/or elevated pulmonary capillary wedge pressure.[37]

Applicant further emphasizes that authors from Atz (Dr. D. Wessel) and Macrae et al[38] (Dr. D Macrae), references cited by the Examiner in the instant Office Action, were members of the Steering Committee that assisted in the drafting of the original protocol, but as evidenced by the exclusion criteria of the original INOT22 protocol described in the Declaration of Dr. Baldassarre, did not anticipate or predict that patients with pre-existing LVD would be at risk of adverse events or serious adverse events arising from treatment with iNO.

After early enrollment of the first 24 subjects in INOT22, an unexpected number of cardiovascular adverse events caused INO and the Steering Committee to amend the exclusion criteria of the INOT22 trial protocol to thereafter exclude patients with pre-existing LVD.[39] Upon conclusion of the INOT22 study and completion of the final study report, INO noted that subsequent to excluding patients with pre-existing LVD, the rate

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[34] Id. at ¶¶7-8.
[35] Id. at ¶9.
[36] Id. at ¶10.
[37] Id. at ¶11.
[38] Cited by the Examiner on page 5 of the Office Action with respect to the state of, or amount of knowledge in the prior art at the time of the invention.
[39] Baldassarre Declaration at ¶12.

of serious adverse events (including serious adverse events associated with heart failure) was significantly reduced.[40]

Based upon this unexpected finding, the FDA approved an amendment to the prescribing information for INOMAX® (nitric oxide) for inhalation to include (i) a statement in the Warnings and Precautions section of the INOMAX prescribing information that states "Heart Failure: In patients with pre-existing left ventricular dysfunction, inhaled nitric oxide may increase pulmonary capillary wedge pressure leading to pulmonary edema", and (ii) section 5.4 of the INOMAX prescribing information that states "Patients who had pre-existing left ventricular dysfunction treated with inhaled nitric oxide, even for short durations, experienced serious adverse events (e.g., pulmonary edema)."[41]

Accordingly, as evidenced and supported by the well documented facts above, in view of the prior art, one skilled in the art at the time of the invention would not have predicted or anticipated that patients with pre-existing LVD would be at risk of experiencing adverse events or serious adverse events arising from treatment with iNO.

The relevant disclosures of the NIH manual, Kinsella and Atz have been discussed *supra*. As noted in the Smith Declaration, Bolooki is a textbook teaching of the clinical uses of the intra-aortic balloon pump, which is not indicated for use in neonates, only adults.[42] Thus, Bolooki is irrelevant to or teaches away from the instantly claimed invention directed to a patient population including neonates or near-term neonates.

As explained in the Smith Declaration, Loh is a study of 19 **adult** patients with an average age of 52 +/- 3 years suffering from ischemic cardiomyapathy (heart failure due to coronary artery disease and resultant partial cardiac muscle death) and idiopathic dilated cardiomyopathy, which resulted in left ventricular failure.[43] The patients also had reactive pulmonary artery HTN secondary to LV failure. The patients were identified as

[40] Id. at ¶13-14.
[41] Id. at ¶¶15-15. See also, Appendix A.
[42] See ¶51.
[43] See ¶49.

having heart failure due to LV dysfunction as classified by NYHA classifications (class III and class IV). They were not identified as having LV dysfunction as it relates to PCWP or PAWP[44]. The study found that in patients with heart failure due to LV dysfunction, inhalation of NO causes a decrease in the PVR associated with an increase in LV filling pressure. These findings predict that iNO, if used alone, may have adverse effects in patients with LV failure.[45] There is no mention, however, that patients with LVD as characterized by an elevated PCWP or PAWP should not receive iNO.

Importantly, the Loh study only identified LVD in **older adults, not neonates or near-term neonates**. As noted in the Smith Declaration, it is well established in the pediatric pulmonary field that iNO clinical studies involving adults cannot be extrapolated to children, neonates or near-term neonates.[46] Thus, the teachings of Loh teach away from or are irrelevant to the instantly claimed invention directed to a patient population including neonates or near-term neonates.

The USPTO recently issued a notice entitled "Examination Guidelines Update: Development in the Obviousness Inquiry After KSR v. Teleflex.[47] The Office Action attempts to combine five prior art references to render claims 1-19 *prima facie* obvious arguing that (apparently in so far as independent claims 2 and 11 are concerned) Atz teaches all of the claim elements except for "expressly teach[ing] a method of excluding patients from treatment with a pulmonary capillary wedge pressure greater than 20 mm Hg."[48] The Office Action asserts that such "deficiency in Atz et al. is cured" by Kinsella, Loh, NIH manual and "common sense."[49]

The Office Action further asserts that

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[44] Pulmonary Capillary Wedge Pressure (PCWP) and Pulmonary Artery Wedge Pressure (PAWP) are synonymous.
[45] Smith Declaratioin at ¶49.
[46] See ¶42-46.
[47] Federal Register, Vol. 75, No. 169 (September 1, 2010), pp. 53643-53660 at 53643. ("SUPPLEMENTARY INFORMATION: 1. *Introduction*. The purpose of this 2010 KSR Guidelines Update is to remind Office personnel of the principles of obviousness explained by the Supreme Court in KSR Int'l Co. v. Teleflex Inc., 550 U.S. 398 (2007 (KSR), and to provide additional guidance in view of decisions by the United States Court of Appeals for the Federal Circuit (Federal Circuit) since KSR."). (Hereinafter referred to as "KSR Guidelines").
[48] Page 15.
[49] Id.

> it is no stretch of the imagination to exclude patients with left ventricular dysfunction with or without the myriad number of other conditions characterized by various medical parameters claimed by Applicant from inhaled nitric oxide therapy in order to avoid adverse outcomes taught by Atz et al. which intrinsically include all the adverse events recited by Applicant. The ordinary artisan would err on the side of caution for the benefit of the patient. Such patients intrinsically have a pulmonary capillary wedge pressure of greater than 20 mm Hg. Inhaled NO increased the wedge pressure as taught by Loh et al. (see entire document) Furthermore, it is merely a design choice by the ordinary artisan to select a ventilator to administer the gas. This is just common sense.[50]

The Office Action then summarily concludes that "one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention."[51]

In response to the Office Action as to independent claims 1, 2 and 11, none of the references mention or recognize using pre-existing LVD, elevated PCWP, or PCWP>20 mmHg as exclusionary criteria for administering iNO for treatment or diagnosis.[52] Moreover, as set forth in the Baldassarre Declaration, prior to the instant filing date, a Steering Committee of internationally recognized experts in the field of pediatric heart and lung disease also did not recognize that pre-existing LVD, elevated PCWP or PCWP>20 mmHg should be exclusionary criteria for iNO.[53]

The fact that Atz and the NIH manual teach cautionary contraindications for iNO (regardless of the patient population) is a teaching away[54] from the instantly claimed invention as it would deny some patients potentially life-saving iNO treatment.[55] Clearly, the claimed exclusionary criteria and attendant medical science would not be (and, in

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[50] Page 16.
[51] Id. at page 17.
[52] See KSR Guidelines at 53648, Example 4.4, citing, Ecolab, Inc. v. FMC Corp., 569 F.3d 1335 (Fed. Cir. 2009) ("Teaching point: A combination of known elements would have been *prima facie* obvious if an ordinarily skilled artisan would have recognized an apparent reason to combine those elements and would have known how to do so.") (emphasis added).
[53] See ¶¶7, 8 and 11.
[54] See MPEP 2141.02.
[55] Smith Declaration at ¶22. See also, KSR Guidelines at 53647, Example 4.2, citing, Crocs, Inc. v. U.S. International Trade Commission, 598 F.3d 1294 (Fed.Cir. 2010). ("Teaching point: A claimed combination of prior art elements may be nonobvious where the prior art teaches away from the claimed combination and the combination yields more than predictable results.").

fact, were not) considered predictable in light of the prior art or a matter of common sense.[56]

As argued *supra*, Loh, the NIH manual and Bolooki are all irrelevant or teach away from the instantly claimed invention because they deal with adult patients, which cannot be correlated or extrapolated to neonates or near-term neonates. And, as argued *supra*, Kinsella fails to recognize or otherwise suggest the instantly claimed acts of excluding neonates and near-term neonates having pre-existing neonates from receiving iNO, including such patients having elevated PCWP or a PCWP>20 mmHg.

Moreover, as set forth in the Smith Declaration, the Committee on Fetus and Newborns of the American Academy of Pediatrics also did not recognize or appreciate the life-saving benefits of excluding neonates or near-term neonates having pre-existing LVD, elevated PCWP or PCWP>20 mmHg from receiving iNO treatment.[57] Still further, prior art cited in the Office Action discussed *supra* (*Macrae, et al., Intensive Care Medicine,* 2004), demonstrates that the European Society of Pediatric Research and the European Society of Neonatology also failed to recognize or appreciate the same life-saving benefits of excluding neonates or near-term neonates having pre-existing LVD, elevated PCWP or PCWP>20 mmHg from receiving iNO treatment.[58]

In sum, the fact that numerous internationally recognized experts as well as the foremost US and European medical pediatric committees were silent about using elevated PCWP or PCWP>20 mmHg as exclusionary criteria for treating neonates or

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[56] Smith Declaration at ¶¶23 and 28. See also, KSR Guidelines at 53648, Example 4.5, citing, Wyers v. Master Lock Co., No. 2009__1412, __F.3d__, 2010 WL 2901839 (Fed.Cir. July 22, 2010). ("Teaching point: The scope of analogous art is to be construed broadly and includes references that are reasonably pertinent to the problem that the inventor was trying to solve. Common sense may be used to support a legal conclusion of obviousness so long as it is explained with sufficient reasoning."). See also, KSR Guidelines at 53649, Example 4.6, citing DePuy Spine, Inc. v. Medtronic Sofamor Danek, Inc., 567 F.3d 1314 (Fed.Cir. 2009). ("Teaching point: Predictability as discussed in KSR encompasses the expectation that prior art elements are capable of being combined, as well as the expectation that the combination that the combination would not have obvious. An inference that the claimed combination would not have been obvious is especially strong where the prior art's teachings undermine the very reason being proffered as to why a person of ordinary skill would have combined the known elements.").

[57] Id. at ¶¶36-41.

[58] Id. at ¶52.

near-term neonates with pre-existing LVD is compelling evidenciary indicia of nonobviousness.[59]

By use of the recitation "intrinsically" in the Office Action, Applicant presumes that the Examiner means inherently. As discussed *supra*, the affirmatively claimed act of excluding neonates or near-term neonates having pre-existing LVD, elevated PCWP or a PCWP>20 mmHg is not disclosed in any of the prior art of record.

Thus, for the foregoing reasons, *inter alia*, independent claims 1, 2 and 11 claims 3-10 and 12-19 depending there from, respectively, are patentably nonobvious over the five cited prior art references. Reconsideration and withdrawal of the rejection is respectfully requested.

Claim 1 is further patentably nonobvious over the five prior art references cited in the Office Action. As argued *supra*, Atz and the NIH manual teach a cautionary contraindication for iNO, which teaches away from an exclusionary contraindication. The NIH manual is also irrelevant or teaches away since it applies to adults, which cannot be correlated to iNO treatment in neonates or near-term neonates. Loh and Bolooki are also irrelevant or teach away because they involve solely adult studies and approved uses. And, Kinsella is silent concerning the affirmative act of neonates or near-term neonates with pre-existing LVD being excluded from treatment with iNO. Thus, there is no *prima facie* case of obviousness in view of the five references cited in the Office Action.

Alternatively, even, *assuming arguendo*, there exists a *prima facie* case of obviousness, as set forth in the Smith and Baldassarre Declarations discussed *supra*, there is ample evidence of indicia of nonobviousness. Reconsideration and withdrawal of the obviousness rejection is respectfully requested.

[59] Smith Declaration at ¶¶41 and 52. See also, Baldassarre Declaration at ¶17. See also, KSR Guidelines at 53657, Example 5.2, citing In re Sullivan, 498 F.3d 1315 (Fed.Cir. 2007). ("Teaching point: All evidence rebutting a prima facie case of obviousness, must be considered when properly presented."). See also, MPEP 716.

Person of Ordinary Skill in the Art

As is well known, the determination of obviousness, while a question of law, is based on underlying factual inquiries that include the level of ordinary skill in the pertinent art. Factors that may be considered are (i) the educational level of the inventor, (ii) the type of problems encountered in the art, (iii) prior art solutions to those problems; (iv) the rapidity with which innovations are made; (v) the sophistication of the technology; and (vi) the education level of active workers in the field. Applicant notes that the members of the Steering Committee were retained because they were well-recognized **experts** in the field, not because they were considered to have "ordinary skill in the art". Thus, although the Steering Committee did not predict or anticipate the risk of adverse events or serious adverse events associated with the use of iNO in study subjects with pre-existing LVD, this level of anticipation is not representative of those of ordinary skill in the art, but rather of those of presumptively **extraordinary** skill. Thus, it is all that much more surprising, and supportive of the non-obviousness of the claims, that the Steering Committee and INOT did not predict or anticipate the risk of adverse events or serious adverse events associated with the use of iNO in patients with pre-existing LVD.

Provisional Obviousness Type Double Patenting Rejections

At pages 17-19 of the Office Action, claims 1-19 stand provisionally rejected on the ground of obviousness type double patenting (OTDP) over claims 1-30 of Application Serial No. 12/494,598, which has been expressly abandoned, thus, rendering this OTDP rejection moot. Reconsideration and withdrawal of the rejection is respectfully requested.[60]

At pages 19-20 of the Office Action, claims 1-19 stand provisionally rejected on the ground of nonstatutory OTDP over claims 1-19 of co-pending Application Serial No. 12/820,980. Applicant respectfully traverses the rejection and the assertions thereof. However, Applicant will file a terminal disclaimer upon allowance of instant application.

[60] See Notice of Abandonment dated September 10, 2010 in Serial No. 12/494,598.

At page 20 of the Office action, claims 1-19 stand provisionally rejected on the ground of nonstatutory OTDP over claims 1-20 of co-pending Application Serial No. 12/821,020. Applicant respectfully traverses the rejection and the assertions thereof. However, Applicant will file a terminal disclaimer upon allowance of instant application.

At page 21 of the Office action, claims 1-19 stand provisionally rejected on the ground of nonstatutory OTDP over claims 1-20 of co-pending Application Serial No. 12/821,041. Applicant respectfully traverses the rejection and the assertions thereof. However, Applicant will file a terminal disclaimer upon allowance of instant application.

In conclusion, Applicant submits that the application is in condition for allowance and respectfully request the same. Examiner Arnold is invited to contact the undersigned to discuss any allowable subject matter or any other matter. No extension of time is needed.

Respectfully Submitted,

Lee & Hayes, PLLC
Representative for Applicant

/Christopher P. Rogers, Reg. No. 36,334/ Dated: September 17, 2010
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Appendix A

INOmax® (nitric oxide) for inhalation

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use INOmax safely and effectively. See full prescribing information for INOmax.

INOmax (nitric oxide) for inhalation
Initial U.S. Approval: 1999

RECENT MAJOR CHANGES

Warnings and Precautions, Heart Failure (5.4) 8/2009

INDICATIONS AND USAGE

INOmax is a vasodilator, which, in conjunction with ventilatory support and other appropriate agents, is indicated for the treatment of term and near-term (>34 weeks gestation) neonates with hypoxic respiratory failure associated with clinical or echocardiographic evidence of pulmonary hypertension, where it improves oxygenation and reduces the need for extracorporeal membrane oxygenation (1.1).

Monitor for PaO_2, methemoglobin, and inspired NO_2 during INOmax administration (1.1).

Utilize additional therapies to maximize oxygen delivery (1.1).

DOSAGE AND ADMINISTRATION

Dosage: The recommended dose of INOmax is 20 ppm, maintained for up to 14 days or until the underlying oxygen desaturation has resolved (2.1).

Administration:

- INOmax must be delivered via a system which does not cause generation of excessive inhaled nitrogen dioxide (2.2).
- Do not discontinue INOmax abruptly (2.2).

DOSAGE FORMS AND STRENGTHS

INOmax (nitric oxide) is a gas available in 100 ppm and 800 ppm concentrations.

CONTRAINDICATIONS

Neonates known to be dependent on right-to-left shunting of blood (4).

WARNINGS AND PRECAUTIONS

Rebound: Abrupt discontinuation of INOmax may lead to worsening oxygenation and increasing pulmonary artery pressure (5.1).

Methemoglobinemia: Methemoglobin increases with the dose of nitric oxide; following discontinuation or reduction of nitric oxide, methemoglobin levels return to baseline over a period of hours (5.2).

Elevated NO_2 Levels: NO_2 levels should be monitored (5.3).

Heart Failure: In patients with pre-existing left ventricular dysfunction, inhaled nitric oxide may increase pulmonary capillary wedge pressure leading to pulmonary edema (5.4).

ADVERSE REACTIONS

Methemoglobinemia and elevated NO_2 levels are dose dependent adverse events. Worsening oxygenation and increasing pulmonary artery pressure occur if INOmax is discontinued abruptly. Other adverse reactions that occurred in more than 5% of patients receiving INOmax in the CINRGI study were: thrombocytopenia, hypokalemia, bilirubinemia, atelectasis, and hypotension (6).

To report SUSPECTED ADVERSE REACTIONS, contact INO Therapeutics at 1-877-566-9466 and http://www.inomax.com/ or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

DRUG INTERACTIONS

Nitric oxide donor agents: Nitric oxide donor compounds, such as prilocaine, sodium nitroprusside, and nitroglycerin, when administered as oral, parenteral, or topical formulations, may have an additive effect with INOmax on the risk of developing methemoglobinemia (7).

Revised: August 2009

FULL PRESCRIBING INFORMATION: CONTENTS*

*Sections or subsections omitted from the full prescribing information are not listed.

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

1.1 Treatment of Hypoxic Respiratory Failure

INOmax® is a vasodilator, which, in conjunction with ventilatory support and other appropriate agents, is indicated for the treatment of term and near-term (>34 weeks) neonates with hypoxic respiratory failure associated with clinical or echocardiographic evidence of pulmonary hypertension, where it improves oxygenation and reduces the need for extracorporeal membrane oxygenation.

Utilize additional therapies to maximize oxygen delivery. In patients with collapsed alveoli, additional therapies might include surfactant and high-frequency oscillatory ventilation.

The safety and effectiveness of inhaled nitric oxide have been established in a population receiving other therapies for hypoxic respiratory failure, including vasodilators, intravenous fluids, bicarbonate therapy, and mechanical ventilation. Different dose regimens for nitric oxide were used in the clinical studies *[see Clinical Studies (14)]*.

Monitor for PaO_2, methemoglobin, and inspired NO_2 during INOmax administration.

2 DOSAGE AND ADMINISTRATION

2.1 Dosage

Term and near-term neonates with hypoxic respiratory failure

The recommended dose of INOmax is 20 ppm. Treatment should be maintained up to 14 days or until the underlying oxygen desaturation has resolved and the neonate is ready to be weaned from INOmax therapy.

An initial dose of 20 ppm was used in the NINOS and CINRGI trials. In CINRGI, patients whose oxygenation improved with 20 ppm were dose-reduced to 5 ppm as tolerated at the end of 4 hours of treatment. In the NINOS trial, patients whose oxygenation failed to improve on 20 ppm could be increased to 80 ppm, but those patients did not then improve on the higher dose. As the risk of methemoglobinemia and elevated NO_2 levels increases significantly when INOmax is administered at doses >20 ppm, doses above this level ordinarily should not be used.

2.2 Administration

The nitric oxide delivery systems used in the clinical trials provided operator-determined concentrations of nitric oxide in the breathing gas, and the concentration was constant throughout the respiratory cycle. INOmax must be delivered through a system with these characteristics and which does not cause generation of excessive inhaled nitrogen dioxide. The INOvent® system and other systems meeting these criteria were used in the clinical trials. In the ventilated neonate, precise monitoring of inspired nitric oxide and NO_2 should be instituted, using a properly calibrated analysis device with alarms. The system should be calibrated using a precisely defined calibration mixture of nitric oxide and nitrogen dioxide, such as INOcal®. Sample gas for analysis should be drawn before the Y-piece, proximal to the patient. Oxygen levels should also be measured.

In the event of a system failure or a wall-outlet power failure, a backup battery power supply and reserve nitric oxide delivery system should be available.

Do not discontinue INOmax abruptly, as it may result in an increase in pulmonary artery pressure (PAP) and/or worsening of blood oxygenation (PaO_2). Deterioration in oxygenation and elevation in PAP may also occur in children with no apparent response to INOmax. Discontinue/wean cautiously.

3 DOSAGE FORMS AND STRENGTHS

Nitric oxide is a gas available in 100 ppm and 800 ppm concentrations.

4 CONTRAINDICATIONS

INOmax is contraindicated in the treatment of neonates known to be dependent on right-to-left shunting of blood.

5 WARNINGS AND PRECAUTIONS

5.1 Rebound

Abrupt discontinuation of INOmax may lead to worsening oxygenation and increasing pulmonary artery pressure.

5.2 Methemoglobinemia

Methemoglobinemia increases with the dose of nitric oxide. In clinical trials, maximum methemoglobin levels usually were reached approximately 8 hours after initiation of inhalation, although methemoglobin levels have peaked as late as 40 hours following initiation of INOmax therapy. In one study, 13 of 37 (35%) of neonates treated with INOmax 80 ppm had methemoglobin levels exceeding 7%. Following discontinuation or reduction of nitric oxide, the methemoglobin levels returned to baseline over a period of hours.

5.3 Elevated NO_2 Levels

In one study, NO_2 levels were <0.5 ppm when neonates were treated with placebo, 5 ppm, and 20 ppm nitric oxide over the first 48 hours. The 80 ppm group had a mean peak NO_2 level of 2.6 ppm.

5.4 Heart Failure

Patients who had pre-existing left ventricular dysfunction treated with inhaled nitric oxide, even for short durations, experienced serious adverse events (e.g., pulmonary edema).

6 ADVERSE REACTIONS

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice. The adverse reaction information from the clinical studies does, however, provide a basis for identifying the adverse events that appear to be related to drug use and for approximating rates.

6.1 Clinical Trials Experience

Controlled studies have included 325 patients on INOmax doses of 5 to 80 ppm and 251 patients on placebo. Total mortality in the pooled trials was 11% on placebo and 9% on INOmax, a result adequate to exclude INOmax mortality being more than 40% worse than placebo.

In both the NINOS and CINRGI studies, the duration of hospitalization was similar in INOmax and placebo-treated groups.

From all controlled studies, at least 6 months of follow-up is available for 278 patients who received INOmax and 212 patients who received placebo. Among these patients, there was no evidence of an adverse effect of treatment on the need for rehospitalization, special medical services, pulmonary disease, or neurological sequelae.

In the NINOS study, treatment groups were similar with respect to the incidence and severity of intracranial hemorrhage, Grade IV hemorrhage, periventricular leukomalacia, cerebral infarction, seizures requiring anticonvulsant therapy, pulmonary hemorrhage, or gastrointestinal hemorrhage.

The table below shows adverse reactions that occurred in at least 5% of patients receiving INOmax in the CINRGI study with event rates >5% and greater than placebo event rates. None of the differences in these adverse reactions were statistically significant when inhaled nitric oxide patients were compared to patients receiving placebo.

Table 1:
Adverse Reactions in the CINRGI Study

Adverse Event	Placebo (n=89)	Inhaled NO (n=97)
Hypotension	9 *(10%)*	13 *(13%)*
Withdrawal	9 *(10%)*	12 *(12%)*
Atelectasis	8 *(9%)*	9 *(9%)*
Hematuria	5 *(6%)*	8 *(8%)*
Hyperglycemia	6 *(7%)*	8 *(8%)*
Sepsis	2 *(2%)*	7 *(7%)*
Infection	3 *(3%)*	6 *(6%)*
Stridor	3 *(3%)*	5 *(5%)*
Cellulitis	0 *(0%)*	5 *(5%)*

6.2 Post-Marketing Experience

The following adverse reactions have been identified during post-approval use of INOmax. Because these reactions are reported voluntarily from a population of uncertain size, it is not always possible to estimate their frequency reliably or to establish a causal relationship to drug exposure. The listing is alphabetical: dose errors associated with the delivery system; headaches associated with environmental exposure of INOmax in hospital staff; hypotension associated with acute withdrawal of the drug; hypoxemia associated with acute withdrawal of the drug; pulmonary edema in patients with CREST syndrome.

7 DRUG INTERACTIONS

No formal drug-interaction studies have been performed, and a clinically significant interaction with other medications used in the treatment of hypoxic respiratory failure cannot be excluded based on the available data. INOmax has been administered with tolazoline, dopamine, dobutamine, steroids, surfactant, and high-frequency ventilation. Although there are no study data to evaluate the possibility, nitric oxide donor compounds, including sodium nitroprusside and nitroglycerin, may have an additive effect with INOmax on the risk of developing methemoglobinemia. An association between prilocaine and an increased risk of methemoglobinemia, particularly in infants, has specifically been described in a literature case report. This risk is present whether the drugs are administered as oral, parenteral, or topical formulations.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category C

Animal reproduction studies have not been conducted with INOmax. It is not known if INOmax can cause fetal harm when administered to a pregnant woman or can affect reproductive capacity. INOmax is not intended for adults.

8.2 Labor and Delivery

The effect of INOmax on labor and delivery in humans is unknown.

8.3 Nursing Mothers

Nitric oxide is not indicated for use in the adult population, including nursing mothers. It is not known whether nitric oxide is excreted in human milk.

8.4 Pediatric Use

Nitric oxide for inhalation has been studied in a neonatal population (up to 14 days of age). No information about its effectiveness in other age populations is available.

8.5 Geriatric Use

Nitric oxide is not indicated for use in the adult population.

10 OVERDOSAGE

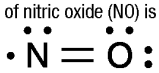
Overdosage with INOmax will be manifest by elevations in methemoglobin and pulmonary toxicities associated with inspired NO_2. Elevated NO_2 may cause acute lung injury. Elevations in methemoglobinemia reduce the oxygen delivery capacity of the circulation. In clinical studies, NO_2 levels >3 ppm or methemoglobin levels >7% were treated by reducing the dose of, or discontinuing, INOmax.

Methemoglobinemia that does not resolve after reduction or discontinuation of therapy can be treated with intravenous vitamin C, intravenous methylene blue, or blood transfusion, based upon the clinical situation.

11 DESCRIPTION

INOmax (nitric oxide gas) is a drug administered by inhalation. Nitric oxide, the active substance in INOmax, is a pulmonary vasodilator. INOmax is a gaseous blend of nitric oxide and nitrogen (0.08% and 99.92%, respectively for 800 ppm; 0.01% and 99.99%, respectively for 100 ppm). INOmax is supplied in aluminum cylinders as a compressed gas under high pressure (2000 pounds per square inch gauge [psig]).

The structural formula of nitric oxide (NO) is shown below:



12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Nitric oxide is a compound produced by many cells of the body. It relaxes vascular smooth muscle by binding to the heme moiety of cytosolic guanylate cyclase, activating guanylate cyclase and increasing intracellular levels of cyclic guanosine 3',5'-monophosphate, which then leads to vasodilation. When inhaled, nitric oxide selectively dilates the pulmonary vasculature, and because of efficient scavenging by hemoglobin, has minimal effect on the systemic vasculature.

INOmax appears to increase the partial pressure of arterial oxygen (PaO_2) by dilating pulmonary vessels in better ventilated areas of the lung, redistributing pulmonary blood flow away from lung regions with low ventilation/perfusion (V/Q) ratios toward regions with normal ratios.

12.2 Pharmacodynamics

Effects on Pulmonary Vascular Tone in PPHN

Persistent pulmonary hypertension of the newborn (PPHN) occurs as a primary developmental defect or as a condition secondary to other diseases such as meconium aspiration syndrome (MAS), pneumonia, sepsis, hyaline membrane disease, congenital diaphragmatic hernia (CDH), and pulmonary hypoplasia. In these states, pulmonary vascular resistance (PVR) is high, which results in hypoxemia secondary to right-to-left shunting of blood through the patent ductus arteriosus and foramen ovale. In neonates with PPHN, INOmax improves oxygenation (as indicated by significant increases in PaO_2).

12.3 Pharmacokinetics

The pharmacokinetics of nitric oxide has been studied in adults.

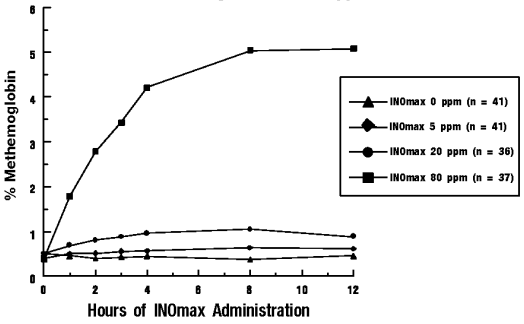
12.4 Pharmacokinetics: Uptake and Distribution

Nitric oxide is absorbed systemically after inhalation. Most of it traverses the pulmonary capillary bed where it combines with hemoglobin that is 60% to 100% oxygen-saturated. At this level of oxygen saturation, nitric oxide combines predominantly with oxyhemoglobin to produce methemoglobin and nitrate. At low oxygen saturation, nitric oxide can combine with deoxyhemoglobin to transiently form nitrosylhemoglobin, which is converted to nitrogen oxides and methemoglobin upon exposure to oxygen. Within the pulmonary system, nitric oxide can combine with oxygen and water to produce nitrogen dioxide and nitrite, respectively, which interact with oxyhemoglobin to produce methemoglobin and nitrate. Thus, the end products of nitric oxide that enter the systemic circulation are predominantly methemoglobin and nitrate.

12.5 Pharmacokinetics: Metabolism

Methemoglobin disposition has been investigated as a function of time and nitric oxide exposure concentration in neonates with respiratory failure. The methemoglobin (MetHb) concentration-time profiles during the first 12 hours of exposure to 0, 5, 20, and 80 ppm INOmax are shown in Figure 1.

Figure 1:
Methemoglobin Concentration – Time Profiles
Neonates Inhaling 0, 5, 20 or 80 ppm INOmax



Methemoglobin concentrations increased during the first 8 hours of nitric oxide exposure. The mean methemoglobin level remained below 1% in the placebo group and in the 5 ppm and 20 ppm INOmax groups, but reached approximately 5% in the 80 ppm INOmax group. Methemoglobin levels >7% were attained only in patients receiving 80 ppm, where they comprised 35% of the group. The average time to reach peak methemoglobin was 10 ± 9 (SD) hours (median, 8 hours) in these 13 patients, but one patient did not exceed 7% until 40 hours.

12.6 Pharmacokinetics: Elimination

Nitrate has been identified as the predominant nitric oxide metabolite excreted in the urine, accounting for >70% of the nitric oxide dose inhaled. Nitrate is cleared from the plasma by the kidney at rates approaching the rate of glomerular filtration.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

No evidence of a carcinogenic effect was apparent, at inhalation exposures up to the recommended dose (20 ppm), in rats for 20 hr/day for up to two years. Higher exposures have not been investigated.

Nitric oxide has demonstrated genotoxicity in Salmonella (Ames Test), human lymphocytes, and after *in vivo* exposure in rats. There are no animal or human studies to evaluate nitric oxide for effects on fertility.

14 CLINICAL STUDIES

14.1 Treatment of Hypoxic Respiratory Failure (HRF)

The efficacy of INOmax has been investigated in term and near-term newborns with hypoxic respiratory failure resulting from a variety of etiologies. Inhalation of INOmax reduces the oxygenation index (OI= mean airway pressure in cm H_2O × fraction of inspired oxygen concentration [FiO_2]× 100 divided by systemic arterial concentration in mm Hg [PaO_2]) and increases PaO_2 *[see Clinical Pharmacology (12.1)]*.

NINOS Study

The Neonatal Inhaled Nitric Oxide Study (NINOS) group conducted a double-blind, randomized, placebo-controlled, multicenter trial in 235 neonates with hypoxic respiratory failure. The objective of the study was to determine whether inhaled nitric oxide would reduce the occurrence of death and/or initiation of extracorporeal membrane oxygenation (ECMO) in a prospectively defined cohort of term or near-term neonates with hypoxic respiratory failure unresponsive to conventional therapy. Hypoxic respiratory failure was caused by meconium aspiration syndrome (MAS; 49%), pneumonia/sepsis (21%), idiopathic primary pulmonary hypertension of the newborn (PPHN; 17%), or respiratory distress syndrome (RDS; 11%). Infants ≤14 days of age (mean, 1.7 days) with a mean PaO_2 of 46 mm Hg and a mean oxygenation index (OI) of 43 cm H_2O / mm Hg were initially randomized to receive 100% O_2 with (n=114) or without (n=121) 20 ppm nitric oxide for up to 14 days. Response to study drug was defined as a change from baseline in PaO_2 30 minutes after starting treatment (full response = >20 mm Hg, partial = 10–20 mm Hg, no response = <10 mm Hg). Neonates with a less than full response were evaluated for a response to 80 ppm nitric oxide or control gas. The primary results from the NINOS study are presented in Table 2.

Table 2:
Summary of Clinical Results from NINOS Study

	Control (n=121)	NO (n=114)	P value
Death or ECMO*,†	77 (64%)	52 (46%)	0.006
Death	20 (17%)	16 (14%)	0.60
ECMO	66 (55%)	44 (39%)	0.014

* Extracorporeal membrane oxygenation

† Death or need for ECMO was the study's primary end point

Although the incidence of death by 120 days of age was similar in both groups (NO, 14%; control, 17%), significantly fewer infants in the nitric oxide group required ECMO compared with controls (39% vs. 55%, p = 0.014). The combined incidence of death and/or initiation of ECMO showed a significant advantage for the nitric oxide treated group (46% vs. 64%, p = 0.006). The nitric oxide group also had significantly greater increases in PaO_2 and greater decreases in the OI and the alveolar-arterial oxygen gradient than the control group (p<0.001 for all parameters). Significantly more patients had at least a partial response to the initial administration of study drug in the nitric oxide group (66%) than the control group (26%, p<0.001). Of the 125 infants who did not respond to 20 ppm nitric oxide or control, similar percentages of NO-treated (18%) and control (20%) patients had at least a partial response to 80 ppm nitric oxide for inhalation or control drug, suggesting a lack of additional benefit for the higher dose of nitric oxide. No infant had study drug discontinued for toxicity. Inhaled nitric oxide had no detectable effect on mortality. The adverse events collected in the NINOS trial occurred at similar incidence rates in both treatment groups *[see Adverse Reactions (6.1)]*. Follow-up exams were performed at 18–24 months for the infants enrolled in this trial. In the infants with available follow-up, the two treatment groups were similar with respect to their mental, motor, audiologic, or neurologic evaluations.

CINRGI Study

This study was a double-blind, randomized, placebo-controlled, multicenter trial of 186 term and near-term neonates with pulmonary hypertension and hypoxic respiratory failure. The primary objective of the study was to determine whether INOmax would reduce the receipt of ECMO in these patients. Hypoxic respiratory failure was caused by MAS (35%), idiopathic PPHN (30%), pneumonia/sepsis (24%), or RDS (8%). Patients with a mean PaO_2 of 54 mm Hg and a mean OI of 44 cm H_2O / mm Hg were randomly assigned to receive either 20 ppm INOmax (n=97) or nitrogen gas (placebo; n=89) in addition to their ventilatory support. Patients who exhibited a PaO_2 >60 mm Hg and a pH < 7.55 were weaned to 5 ppm INOmax or placebo. The primary results from the CINRGI study are presented in Table 3.

Table 3:
Summary of Clinical Results from CINRGI Study

	Placebo	INOmax	P value
ECMO*,†	51/89 (57%)	30/97 (31%)	<0.001
Death	5/89 (6%)	3/97 (3%)	0.48

* Extracorporeal membrane oxygenation

† ECMO was the primary end point of this study

Significantly fewer neonates in the INOmax group required ECMO compared to the control group (31% vs. 57%, p<0.001). While the number of deaths were similar in both groups (INOmax, 3%; placebo, 6%), the combined incidence of death and/or receipt of ECMO was decreased in the INOmax group (33% vs. 58%, p<0.001).

In addition, the INOmax group had significantly improved oxygenation as measured by PaO_2, OI, and alveolar-arterial gradient (p<0.001 for all parameters). Of the 97 patients treated with INOmax, 2 (2%) were withdrawn from study drug due to methemoglobin levels >4%. The frequency and number of adverse events reported were similar in the two study groups *[see Adverse Reactions (6.1)]*.

14.2 Ineffective in Adult Respiratory Distress Syndrome (ARDS)

ARDS Study

In a randomized, double-blind, parallel, multicenter study, 385 patients with adult respiratory distress syndrome (ARDS) associated with pneumonia (46%), surgery (33%), multiple trauma (26%), aspiration (23%), pulmonary contusion (18%), and other causes, with PaO_2/FiO_2 <250 mm Hg despite optimal oxygenation and ventilation, received placebo (n=193) or INOmax (n=192), 5 ppm, for 4 hours to 28 days or until weaned because of improvements in oxygenation. Despite acute improvements in oxygenation, there was no effect of INOmax on the primary endpoint of days alive and off ventilator support. These results were consistent with outcome data from a smaller dose ranging study of nitric oxide (1.25 to 80 ppm). INOmax is not indicated for use in ARDS.

16 HOW SUPPLIED/STORAGE AND HANDLING

INOmax (nitric oxide) is available in the following sizes:

Size D	Portable aluminum cylinders containing 353 liters at STP of nitric oxide gas in 800 ppm concentration in nitrogen (delivered volume 344 liters) (NDC 64693-002-01)
Size D	Portable aluminum cylinders containing 353 liters at STP of nitric oxide gas in 100 ppm concentration in nitrogen (delivered volume 344 liters) (NDC 64693-001-01)
Size 88	Aluminum cylinders containing 1963 liters at STP of nitric oxide gas in 800 ppm concentration in nitrogen (delivered volume 1918 liters) (NDC 64693-002-02)
Size 88	Aluminum cylinders containing 1963 liters at STP of nitric oxide gas in 100 ppm concentration in nitrogen (delivered volume 1918 liters) (NDC 64693-001-02)

Store at 25°C (77°F) with excursions permitted between 15–30°C (59–86°F) [see USP Controlled Room Temperature].

Occupational Exposure

The exposure limit set by the Occupational Safety and Health Administration (OSHA) for nitric oxide is 25 ppm, and for NO_2 the limit is 5 ppm.

INO Therapeutics
6 Route 173 West
Clinton, NJ 08809
USA

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No. 12/820,866
Filing Date 6/22/2010
Confirmation No. 2913
First Named Inventor ..... James S. Baldassarre
Assignee .....
Group Art Unit 1613
Examiner ..... Ernst V Arnold
Attorney's Docket No. I001-0002USC1
Title: METHODS OF TREATING TERM AND NEAR-TERM NEONATES HAVING HYPOXIC RESPIRATORY FAILURE ASSOCIATED WITH CLINICAL OR ECHOCARDIOGRAPHIC EVIDENCE OF PULMONARY HYPERTENSION

To: Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

From: Christopher P. Rogers (Tel.; Fax 509-323-8979)
Customer Number: 49584
Lee & Hayes, PLLC
601 W. Riverside Avenue, Suite 1400
Spokane, WA 99201

Fees will be paid by credit card through the EFS Web; however the Commissioner is hereby authorized to charge any deficiency of fees and credit any overpayments to Deposit Account Number 12-0769.

Respectfully Submitted,

Dated: 1 October 2010 By: _____
Christopher P. Rogers
Reg. No. 36,334

CURRICULUM VITAE

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EDUCATION

2001- 2004 GONZAGA UNIVERSITY
SCHOOL OF LAW
Spokane, WA
- **Juris Doctorate**

1988- 1990 YALE UNIVERSITY
SCHOOL OF MEDICINE
New Haven, CT
- **Physician Associate**

Honors and Awards:
- 1990 Yale Medical Writing Award
- Education and Research Foundation Scholarship

1984- 1988 WHITWORTH UNIVERSITY
Spokane, WA
- **BS- Health Promotion and Education**

1980- 1982 SPOKANE COMMUNITY COLLEGE
Spokane, WA
- **AAS- Respiratory Therapy**

PROFESSIONAL LEGAL EXPERIENCE

2009- Current LEE & HAYES, PLLC
Spokane, WA
Attorney and Counselor-at-Law
Practice focuses on General Transactional Practice, including Business Law, Contracts, and Estate Planning. In addition, practice includes general intellectual property issues such as Copyright and Trademark.

2006- 2009 THE SMITH LAW GROUP, PLLC
Spokane, WA
Attorney and Counselor-at-Law
Practice focuses on General Transactional Practice, including Business Law, Contracts, and Estate Planning, and also includes Civil Litigation in First Amendment issues, Medical Negligence, and Wrongful Death.

2003- Current ALLIANCE DEFENSE FUND
Scottsdale, AZ
Allied Attorney- Attended Alliance Defense Fund's National Litigation Academy July 2006. Responsibilities include:
- Providing 450 hours of pro bono work over 3 years
- Assisting with litigation in First Amendment cases
- Providing research and assistance to allied organizations

Blackstone Fellow- Commissioned January 2003- Responsibilities include:
- Supporting the work of the Alliance Defense Fund through scholarly research and writing in areas of First Amendment and Equal Protection

2004 SHAUN CROSS FOR CONGRESS- U.S. CONGRESSIONAL CAMPAIGN- WA 05
Spokane, WA
Campaign Coordinator- Responsibilities included:
- Implemented all aspects of political strategy
- Management of campaign staff
- Coordinated, planned, and scheduled all campaign events
- Interfaced between all media and candidate and served as spokesman for the campaign
- Researched and drafted policy paper

2003- 2004 PAINE, HAMBLEN, COFFIN, BROOKE & MILLER, LLP
Spokane, WA
Summer Associate/ 3L Associate- Responsibilities included:
- Legal Research
- Drafting Legal Memoranda
- Client Interview
- Attending and assisting with depositions
- Preparing Oral Arguments
- Participate in Negotiations
- Court Appearance as Rule 9 Intern- Ex Parte, Hearings, Etc.
- Analyzing and preparing Medical Records and Chronologies

2003 EYMANN, ALLISON, FENNESSEY, HUNTER & JONES
Spokane, WA
Consultant- Responsibilities included:
- Medical Record Review
- Providing Expert Medical Opinion
- Producing Written Reports

2002- 2003 LAW OFFICES OF KEN COLEMAN, MD, JD
Spokane, WA
Consultant - Responsibilities included:
- Screening and Interviewing Potential Clients
- Medical Record Review
- Assistance in Case Workup
- Legal Research

2002 LEE & ISSERLIS, PS
Spokane, WA
Consultant- Responsibilities included:
- Medical Record Review
- Observation of Disability Exams and Writing Exam Reports

2002 [summer] BOPP, COLESON, & BOSTROM

Lead Council- National Right to Life Committee
Terre Haute, IN
Blackstone Fellowship Intern- Responsibilities included:

- Worked on the 9th Circuit Court of Appeals case of *Oregon v. Ashcroft*
  - Legal Research
  - Writing memorandum
  - Strategizing for Amicus Brief

PROFESSIONAL MEDICAL EXPERIENCE

1993- 2006 ROCKWOOD CLINIC, PS

DEPARTMENT OF FAMILY MEDICINE AND URGENT CARE
DEPARTMENT OF PLASTIC AND RECONSTRUCTIVE SURGERY
Spokane, WA
PA- Responsibilities include:

- Providing primary and urgent care for a patient population ranging from pediatrics to geriatrics including GYN and orthopedics with a special interest in Sports Medicine.
- Clinical Research Sub-Investigator
- Provide Expert Medial Opinion for Medical-Legal Cases
- Conducted pre operative physical exams
- First Assist in complex plastic and reconstructive surgical cases
- Post operative follow up in clinic and hospital

1990- 1993 CARDIOVASCULAR & THORACIC SURGICAL ASSOCIATES, PS

Spokane, WA
PA- Responsibilities included:

- Saphenous Vein Harvesting
- First Assisting in the OR
- Patient Management in ICU, Critical Care, and Post-Op Unit
- Admission Physicals
- Discharge Summaries
- Case Experience [approximate]
  - 500 CABG and/ or AVR/ MVR
  - 200 Thoracotomies
  - 120 Peripheral Vascular
  - 25 Heart/ Heart-Lung Transplants

1986- 1988 AMERICAN LUNG ASSOCIATION OF WASHINGTON

Spokane, WA
Eastern Washington Regional Director- Responsibilities included:

- Development and implementation of various ALAW Programs
- Management of Staff
- Management of Volunteers

1984- 1988 PACIFIC HEALTH MANAGEMENT, INC
Spokane, WA
Consultant- Responsibilities included:
- Consulting and Training in the areas of Health Promotion, Fitness Testing, Exercise Programming and Prescription, and Human Resource Development

1984- 1986 KOOTENAI MEDICAL CENTER
Coeur d'Alene, ID
Pulmonary Education Coordinator- Responsibilities included:
- Training and In-servicing Medical Staff
- Developing and teaching Pulmonary Rehabilitation Program
- Teaching Smoking Cessation Programs
- Teaching Asthma Education Programs
- Marketing Out-Patient Programs

1982- 1984 LOMA LINDA UNIVERSITY MEDICAL CENTER
Loma Linda, CA
Clinical Supervisor- Neonatal and Pediatric ICU's/ Trauma Team
Responsibilities included:
- All aspects of mid-management such as scheduling, staff evaluations, filing regular reporting forms, and being responsible for all respiratory care delivered in clinical area.

Registered Respiratory Therapist- Neonatal and Pediatric ICU's/ Trauma Team
Responsibilities included:
- General and Critical Respiratory Care
- Transport team member [fixed wing/ helicopter]
- Trauma team member

▣ PROFESSIONAL CREDENTIALS

2007 UNITED STATES COURT OF APPEALS
NINTH CIRCUIT
Admitted March 2007

2007 UNITED STATES DISTRICT COURT
EASTERN DISTRICT OF WASHINGTON
Admitted January 2007

2006 WASHINGTON STATE BAR ASSOCIATION
Admitted to Practice in all State Courts in Washington May 2006
WSBA-ID# 37460

2001 CERTIFIED- TEAM PHYSICIAN COURSE
American College of Sports Medicine

1990 PHYSICIAN ASSISTANT- CERTIFIED
Special Recognition in PRIMARY CARE and SURGERY cores
NCCPA #910950 **Re-certified August 1997**
WA State License # PA2311
DEA # MS0090996

1986 ADVANCED CARDIAC LIFE SUPPORT PROVIDER
American Heart Association
Re- certified 1998, 2002, 2005

1986 CERTIFIED HEALTH/ FITNESS INSTRUCTOR
American College of Sports Medicine
Cert # 792

1984 CERTIFIED PROGRAM INSTRUCTOR
American Institute of Preventive Medicine

1983 RRT- REGISTERED RESPIRATORY THERAPIST
National Board of Respiratory Care
REG # 19756

VOLUNTEER MEDICAL ACTIVITIES

2003- 2004 IRONMAN NORTH AMERICA
Coeur d'Alene, Idaho
Medical Staff

2000- 2005 SPOKANE SHADOW PROFESSIONAL SOCCER TEAM [PDL]
Spokane, WA
Team Medical Staff

1996 NORBA (downhill & XC MTN bike) Championship Series #5
Mt. Spokane, WA
Team Medical Staff

1995 TRANS-PACIFIC SERVICE LEAGUE
Iloilo, Philippines
Medical Missions- Primary Care and Minor Surgery

1993- 1996 BIG SKY TRACK AND FIELD CHAMPIONSHIPS
Eastern Washington University
Cheney, WA
Medical Staff

1993- 1995 HOOPFEST- 3 on 3 BASKETBALL TOURNAMENT
Spokane, WA
Medical Staff

1993- 1994 GATORADE IRONMAN WORLD CHAMPIONSHIP
Kona, Hawaii
Medical Staff

VOLUNTEER LEGAL ACTIVITIES

2007 VOLUNTEER LAWYERS PROGRAM
HOUSING JUSTICE PROJECT
Spokane County Bar Association
Spokane, WA

▣ COMMUNTY VOLUNTEER ACTIVITIES

2006- Current — ALLIES FOR MARRIAGE AND CHILDREN
BOARD OF DIRECTORS, Vice-President
Seattle, WA

2005- Current — LIFE SERVICES OF SPOKANE
BOARD OF DIRECTORS, Chair
Spokane, WA

2009- Current
2006- 2008
2003- 2005
2000- 2002
1997- 1999 — TURNING POINT CHURCH
BOARD OF DIRECTORS [5 TERMS]
Spokane, WA

1998- Current — TURNIGN POINT CHURCH
DIRECTOR- MEN'S MINISTRIES
Spokane, WA

▣ ACADEMIC APPOINTMENTS

1997- Current — UNIVERSITY OF WASHINGTON
SCHOOL OF MEDICINE- MEDEX
Seattle, WA
Adjunct Clinical Faculty/ Lecturer
Excellence in Teaching Award- 2000 and 2001

1999- 2000 — TREVECCA NAZARENE UNIVERSITY
SCHOOL OF HEALTH PROFESSIONS
Nashville, TN
Adjunct Clinical Faculty

1997- 1998 — OREGON HEALTH SCIENCES UNIVERSITY
SCHOOL OF MEDICINE- PA PROGRAM
Portland, OR
Clinical Preceptor

1996- 1997 — WESTERN MICHIGAN UNIVERSITY
COLLEGE OF HEALTH AND HUMAN SERVICES
PHYSICIAN ASSISTANT DEPARTMENT
Kalamazoo, MI
Clinical Preceptor

▣ INVITED LECTURES AND PRESENTATIONS

2000 — WASHINGTON ACADEMY OF PHYSICIAN ASSISTANTS
Annual Fall Conference
Spokane, WA
- TOPIC: *Office Techniques for Smoking Cessation*

1999 AMERICAN ROAD RUNNERS CLUB
National Convention in association with Bloomsday
Spokane, WA
- TOPIC: *Men's and Women's Health Issues- Panel Moderator/Participant*

1998 ARNP's UNITED OF WASHINGTON STATE
13th Annual Primary Care Conference
Spokane, WA
- TOPIC: *Wound Care and Suturing Workshop*

1995 WASHINGTON INTERSCHOLASTIC ACTIVITIES ASSOCIATION
1995 Cheerfest
Spokane, WA
- TOPIC: *The Art of Stretching*

1990 HEALTH UNIT COORDINATORS
Annual Conference- Sacred Heart Medical Center
Spokane, WA
- TOPIC: *Thoracic Transplantation*

MEDIA EXPERIENCE

2009- Current CELEBRATE LIFE RADIO PROGRAM
Co-host weekly half hour radio program highlighting pro-life issues

2006- 2009 CROSSPOINT RADIO PROGRAM
Co-host weekly half hour radio program which airs 6 times each week on the ACN radio network.

2004 CROSS FOR CONGRESS
Spokane, WA
- Served as official spokesman for the campaign and wrote all press releases. Also, wrote *"The Cross Report"* a weekly radio program which aired on the ACN radio network and it's five affiliates across Eastern Washington.

1988 AMERICAN LUNG ASSOCIATION OF WASHINGTON and KHQ TV
Spokane, WA
- Wrote, organized and developed, *"Freedom From Smoking on the Air."* This program ran for 21 consecutive days on the evening news educating viewers how to quit smoking. The program had 12,000 participants and was nominated for the Public Relation Society of America's "Totem Award" for excellence in Public Health Education Programming.

1984- 1988 Personal appearances on:
- KXLY (ABC) "Noon Show" and "Health Beat"
- KHQ (NBC) "Up Front" and "Sunday"
- Local News, Radio, and Newspaper

▣ PUBLICATIONS

Jeffrey R. Smith, *From Knowledge to Action*, Blackstone Fellowship Newsletter, Vol. 2, February 2003.

Rodney Moser, Editor, Jeffrey R. Smith, Contributing Author [Chapters on Valvular Heart Disease (Section 1 Chapter 10) and Heat Stroke (Section 4 Chapter 6), *Primary Care for Physician Assistants*, (1st ed. 1998, 2d ed. 2001).

Rodney Moser, Editor, Jeffrey R. Smith, Contributing Author [Review Questions with Annotated Answers on Valvular Heart Disease and Heat Stroke], *Primary Care for Physician Assistants- Self- Assessment and Review Study Guide*, (1st ed. 1998, 2d ed. 2001).

Smith JR, Reese SA: *Twelve Days in May... a medical missions experience in the Philippines*, TPSL Newsletter, June 1995.

Smith JR, Elefteriades JE: *Ventricular Assist Devices and Their Role in Cardiac Transplantation*, Journal of the American Academy of Physician Assistants, submitted 1990.

Wilkins RL, Dexter JR, Smith JR: *Survey of Adventitious Lung Sound Terminology in Case Reports*, CHEST 85:523-25, 1984.

▣ PROFESSIONAL AND PERSONAL REFERENCES

Shaun Cross
U.S. Congressional Candidate WA-05 [September 2003- September 2004]
CEO, Lee & Hayes, LLP
Spokane, WA
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Special Emissary for the Vatican to the United Nations
Professor of Law, Gonzaga University School of Law
Spokane, WA
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Rev. Monte LeLaCheur
Senior Pastor
Turning Point Open Bible Church
Spokane, WA
509-467-5122

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE (USPTO)	
Application Serial Number	12/820,866
Confirmation Number	2913
Filing Date	June 22, 2010
Title of Application	Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension
First Named Inventor	James S. Baldassarre
Assignee	Ikaria, Inc.
Group Art Unit	1613
Examiner	Arnold, Ernst V.
Attorney Docket Number	I001-0002USC1

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF JEFFREY R. SMITH, ESQ.
UNDER 37 C.F.R. § 1.132

I, Jeffrey R. Smith, Esq., hereby declare the following:

1. I practiced as a nationally Registered Respiratory Therapist (RRT) in the areas of neonatal and pediatric intensive care from 1982-1990, garnering in depth knowledge and experience in the area of cardiovascular and pulmonary physiology. This experience and education is highly and directly relevant to the claimed invention referenced, *infra.*

2. I graduated from Yale University School of Medicine, Physician Associate Program in 1990.

3. From 1990 until 2006 I was a nationally certified PA, duly licensed to practice medicine in the State of Washington, Washington license PA2311.

4. From 1990 until 1993, my practice was with Cardiovascular and Thoracic Surgery Associates, located in Spokane, WA. In addition to first assisting with heart,

lung, and peripheral vascular surgery, my practice also consisted of management of critically ill patients in the cardiac and intensive care units.

5. From 1993-2006 my practice was primarily at Valley Rockwood Clinic, PS, Department of Family and Sports Medicine, Spokane, WA. In addition to primary care, my practice consisted of Urgent Care medicine as well.

6. I graduated from Gonzaga University School of Law in 2004, admitted to practice law in the State of Washington, U.S. District Court Eastern District of Washington, and the Ninth Circuit Court of Appeals. I was in private practice until joining Lee & Hayes, PLLC in September 2009, and am currently employed by Lee & Hayes, PLLC.

7. I have been qualified and have served as an expert in several medically related litigation cases.

8. I am currently not a registered patent attorney before the USPTO.

9. A true and accurate copy of my curriculum vitae is attached as Exhibit A.

10. All citations referred to in this declaration are attached as Exhibit B.

ART RECOGNIZED MEANINGS of ADVERSE EVENT and SERIOUS ADVERSE EVENT

11. In medical practice and research, the phrases "adverse events" and "serious adverse events" have specific, well understood meanings.

12. The FDA, in Federal regulations 21 CFR Part 312, defines "adverse events" as any untoward medical occurrence that may present itself during treatment or administration with a pharmaceutical product, and which may or may not have a causal relationship with the treatment.[1]

13. In the guideline entitled "Clinical Safety Data Management: Definitions and Standards for Expedited Reporting", the FDA further clarifies that an "adverse event" is "serious" (i.e., a "serious adverse event") when the patient outcome results in death; is life-threatening; requires inpatient hospitalization or prolongation of existing

[1] *See*, http://www.gcrc.uci.edu/rsa/aer.cfm

hospitalization; creates persistent or significant disability/incapacity, or a congenital anomaly/birth defects.[2]

14. On August 28, 2009, the FDA approved the addition of a new warning within section 5.4 of the prescribing information for INOMAX (nitric oxide) for inhalation: "patients who had pre-existing left ventricular dysfunction treated with inhaled nitric oxide, even in short durations, experienced **serious adverse events** (e.g., pulmonary edema)" (emphasis added).

15. In sum, it is my expert medical opinion that "adverse event" and "serious adverse event" are well understood and commonly used terms in the medical arts.

ART RECOGNIZED MEANING of PRECAUTION vs. EXCLUSION

16. In the practice of medicine, frequently, there are ideas and concepts which are so basic that finding a specific citation to describe the idea or concept proves challenging. A specific citation from a medical source describing the difference between a *precaution* and *exclusion* is one such concept.

17. Hence, in order to form a framework of understanding and definition of the terms *precaution* and *exclusion* the Encarta Webster's[3] definition is used to describe the plain meaning, and, thus, how these meanings are applied in a medical context.

18. *Precaution* is defined as "the foresight to protect against **possible harm** or trouble or to limit the damage if something goes wrong," or "an action taken to protect against **possible harm** or trouble or to limit the damage if something goes wrong."[4]

19. In contrast, *exclusion* is defined as "to **prevent** somebody or something from entering or participating" or "to **prevent** somebody or something from being considered or accepted."[5]

20. Further, while no specific medical definitions for *precaution* or *exclusion* were identified, application and how these terms are regularly used in medical arts are readily apparent to a medical practitioner of ordinary skill.

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[2] *See*, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm073087.pdf. See also, MedWatch The FDA Safety Information Adverse Event Reporting Program, and 21 CFR 312.32(a).
[3] The Encarta Webster's Dictionary of the English Language (2004) is the second edition of the Encarta World English Dictionary, published in 1999 (Anne Soukhanov, editor).
[4] http://encarta.msn.com/encnet/features/dictionary/dictionaryhome.aspx (emphasis added).
[5] *Id.*(emphasis added).

21. For example, it is my expert medical opinion, based on years of experience, that virtually every case study or report related to medical or pharmacologic research clearly identifies and describes *exclusion* criteria for study subjects.[6] This *exclusion* criteria clearly means that study subjects are prevented from enrolling in the study and are not allowed to participate. One such study identified 17 criteria, any of which prevented participation without exception.[7] Another study, when discussing *exclusion* criteria, was even more clear. It stated, "patients meeting any of the following criteria must not be enrolled in the study."[8]

22. On the contrary, *precautions* allow subject participation and hence **teach away** from the claimed invention of which this declaration is the subject, whereby patients are excluded from potentially beneficial therapy.

23. For the aforementioned reasons, it is my expert medical opinion that any prior art teaching *precautions* concerning administering inhaled nitric oxide (iNO) would not render obvious *exclusions*, which is a subject of the instantly claimed invention. Moreover, it is my expert medical opinion that it is not common sense to jump from a precaution to an exclusion of any medical treatment. It is also my expert medical opinion that selection of exclusionary criteria for children being treated with iNO would not be predictable, obvious to try, common sense or a simple clinical design choice.

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[6] See, *e.g.*, *Dermatological Cryosurgery in Primary Care with Dimethyl Ether Propane Spray in Comparison with Liquid Nitrogen*, Martinez, et al., Atnecion Primaria, Vol. 18 No. 5 (211, 216), September 30, 1996; *Sibutramine-metformin Combination vs. Sibutramine and Metformin Monotherapy in Obese Patients*, July 15, 2009, p. 3, ClinicalTrials.gov, http://clinicaltrials.gov/ct2/show/NCT00941382; *Study of Comparative Effects of Oral Clonidine vs. Oral Diazepam Pre-Medication on the Extent and Duration of Sensory Blockade in Patients Undergoing Vaginal Hysterectomy Under Spinal Anaesthesia*, Toshniwal, et al., Internet Journal of Anesthesiology, 2009, http://www.britannica.com/bps/additionalcontent/18/41575551/ Study-of –Comparative-Effects-of-Oral-Clonidine-vs-Oral-Diazepam-Pre-Medication-on-the-Extent-and-Duration-of-Sensory-Blockade-in-Patients-Undergoing-Vaginal-Hysterectomy-Under-Spinal-Anaesthesia; *Pazopanib Plus Lapatinib Compared to Lapatinib Alone in Subjects With Inflammatory Breast Cancer*, April 22, 2010, p. 4, ClinicalTrials.gov, http://clincialtrials.gov/ct2/show/NCT00558103; *Dronedarone is Less Effective, But Safer Than Amiodarone in Atrial Fibrillation*, October 27, 2009, p. 3, http://www.npci.org.uk/blog/?p=778.

[7] *Sibutramine-metformin Combination vs. Sibutramine and Metformin Monotherapy in Obese Patients*, July 15, 2009, p. 3, ClinicalTrials.gov, http://clinicaltrials.gov/ct2/show/NCT00941382

[8] *Pazopanib Plus Lapatinib Compared to Lapatinib Alone in Subjects With Inflammatory Breast Cancer*, April 22, 2010, p. 4, ClinicalTrials.gov, http://clincialtrials.gov/ct2/show/NCT00558103 (emphasis added).

ART RECOGNIZED MEANINGS of RELATIVE vs. ABSOLUTE CONTRAINDICATIONS

24. In the practice of medicine, substantial nonobvious differences exist between the designations[9] "relative contraindication" and "absolute contraindication."

25. A contraindication is a condition that makes a particular treatment or procedure *inadvisable*. It is well understood by those skilled in the art that a contraindication may be characterized as absolute or relative.

26. An absolute contraindication is a situation that makes a particular treatment or procedure *absolutely inadvisable*. In a baby, for example, aspirin is absolutely contraindicated because of the danger that aspirin will cause Reye syndrome.[10] Stated another way, the risk to the patient absolutely outweighs any potential benefit the patient may gain from a treatment or procedure. Hence, no situation exists in which it would be appropriate to provide such treatment or procedure, and the patient is absolutely denied such treatment.

27. In contrast, a relative contraindication is a condition that makes a particular treatment or procedure somewhat inadvisable but does not rule it out. For example, X-rays in pregnancy are relatively contraindicated (because of concern for the developing fetus) unless the X-ray is absolutely necessary.[11] In many cases, treatment of a pregnant patient's fracture likely outweighs the potential danger imposed upon the developing fetus. In another example, surgery on a patient taking blood thinners is relatively contraindicated, unless the surgery is needed to save the patient's life. The benefit to the patient would outweigh the risk of bleeding. Thus, it would be appropriate to provide the treatment or procedure in spite of the relative contraindication.

28. For the aforementioned reasons, it is my expert medical opinion that any prior art teaching a relative contraindication concerning administration of iNO would not render obvious the instantly claimed invention of absolutely excluding treatment with iNO where the patient has been diagnosed as having pre-existing left ventricular

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[9] Such designations are indicated on the drug product label approved by the FDA.
[10] *See*, MedicineNet.com; http://www.medterms.com/script/main/art.asp?articlekey=17824
[11] *Id.*

dysfunction (LVD), elevated pulmonary capillary wedge pressure (PCWP), or, a PCWP>20 mmHg. Again, as stated *supra*, it is not common sense nor predictable in the medical arts to jump from a relative contraindication to an absolute contraindication for any medical treatment. Moreover, it is my expert medical opinion that selection of absolute contraindication criteria for children being treated with iNO would not be predictable, obvious to try, common sense or a simple clinical design choice.

ART RECOGNIZED DESCRIPTION of METHODS USED to DIAGNOSE LEFT VENTRICULAR FAILURE

29. Ventricular dysfunction is a condition of the heart whereby the ventricular function is diminished, and, hence, results in pump failure. "Heart failure is the pathophysiologic state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood at a rate commensurate with the requirements of the metabolizing tissues...."[12]

30. Ventricular dysfunction may be characterized by a number of factors. Whether the dysfunction affects the right side of the heart or the left side of the heart is one factor to consider when describing and diagnosing ventricular dysfunction.

31. "Many of the clinical manifestations of heart failure result from the accumulation of excess fluid behind either one or both ventricles. For example, patients in whom the left ventricle is mechanically overloaded (e.g., aortic stenosis) or weakened (e.g., post myocardial infarction) develop dyspnea and orthopnea as a result of pulmonary congestion. In contrast, when the underlying abnormality affects the right ventricle primarily (e.g., valvular pulmonic stenosis or pulmonary hypertension secondary to pulmonary thromboembolism), symptoms resulting from pulmonary congestion such as...edema, congestive hepatomegaly, and systemic venous distention...are more common."[13]

32. Diagnosing left ventricular failure is initially accomplished by recognizing physical signs and clinical manifestations such as dyspnea, orthopnea, paroxysmal nocturnal dyspnea, fatigue, weakness, abnormal lung sounds, cardiac edema,

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[12] Harrison's Principles of Internal Medicine, Fauci, et al., p.1287, 12th Edition, McGraw Hill, 1998.
[13] *Id.* at 1288.

hydrothorax, ascites, congestive hepatomegaly, jaundice, and weight loss to name a few.[14]

33. Other tests and indices are helpful in diagnosing left ventricular failure. Chest x-rays showing enlargement of the heart, distention of pulmonary veins, and pleural effusions with associated interlobar effusions may be evident.[15] Cardiac output, ventricular ejection fraction, pulmonary capillary wedge pressure, and echocardiographic evidence of ventricular wall motion are other tests which aid in diagnosing failure of the left ventricle.[16]

34. On August 28, 2009, the FDA approved the addition of a new warning within section 5.4 of the prescribing information for INOMAX® (nitric oxide) for inhalation which states "patients who had pre-existing left ventricular dysfunction treated with inhaled nitric oxide, even in short durations, experienced serious adverse events (e.g., pulmonary edema)." The new language was added to the INOMAX prescribing information after completion of the INOT22 study. The FDA did not limit the scope and language of the warning solely to patients with pre-existing left ventricular dysfunction as diagnosed by a PCWP greater than 20 mmHg, but rather warned against the use of iNO in patients with pre-existing left ventricular dysfunction generally, regardless of the means used to identify, characterize and diagnose pre-existing left ventricular dysfunction.

35. In sum, the use of elevated capillary wedge pressure is but one of many diagnostic measures known to those skilled in the medical arts to identify and diagnose left ventricular dysfunction.

RECOMMENDATIONS of the AMERICAN ACADEMY of PEDIATRICS

36. The American Academy of Pediatrics (AAP), considered by most in the medical field as the pre-eminent organization for pediatricians, was founded in 1930 and now has 60,000 primary care pediatricians, pediatric medical subspecialists and pediatric surgical specialists as members.

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[14] *Id.* at 1289-1291.
[15] *Id.* at 1291.
[16] *Id.* at 1360.

37. The AAP has a variety of committees that focus on subspecialty areas. The Committee on Fetus and Newborns is one such subcommittee.

38. In their August, 2000 edition of Pediatrics, Vol. 106 No. 2, the Committee on Fetus and Newborns produced the committee's recommendations for use of iNO, and conditions under which iNO should be administered to the neonate with hypoxic respiratory failure.[17,18]

39. After a general discussion on the indications, mechanisms of action, and uses of iNO, the Committee specifically outlines seven recommendations under which iNO should be administered to the neonate with hypoxic respiratory failure.[19]

40. Although the recommendations specifically mention monitoring and other testing, such as echocardiaography, the recommendations are absolutely silent regarding exclusion of patients with LVD, elevated PCWP, or PCWP>20 mmHg.[20]

41. It is my expert medical opinion, such silence by the Committee on Fetus and Newborns of the AAP (in their official statement and recommendations under which iNO should be administered to the neonate with hypoxic respiratory failure) concerning exclusion of patients with pre-existing LVD, elevated PCWP or, PCWP>20 mmHg, or pre-existing LVD is compelling evidence of the novelty and nonobviousness of the instantly claimed invention.

LVD in CHILDREN, TERM and NEAR-TERM NEONATES vs. ADULTS

42. In an article by Dr. Steven E. Lipshultz, he examines, analyzes, and discusses the current issues surrounding clinical research of ventricular dysfunction in infants, children, and adolescents. Throughout the article the author makes the distinction that

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[17] American Academy of Pediatrics, Committee on Fetus and Newborn, Use of Inhaled Nitric Oxide, PEDIATRICS Vol. 106 No. 2,p. 344-345, August 2000. See also, See http://www.aap.org/visit/cmte17.htm, Committee on Fetus and Newborn.

[18] This reference is directly on point with the instantly claimed invention which is titled, *Methods of Treating Term and Near-Term Neonates Having* ***Hypoxic Respiratory Failure*** *Associated With Clinical or Echocardiographic Evidence of Pulmonary Hypertension.* (emphasis added).

[19] American Academy of Pediatrics, Committee on Fetus and Newborn, Use of Inhaled Nitric Oxide, PEDIATRICS Vol. 106 No. 2,p. 344-345, August 2000.

[20] *Id.*

children with ventricular dysfunction must be diagnosed, understood, and treated differently than adult patients with ventricular dysfunction.[21]

43. He states, "[m]any changing developmental properties of the pediatric myocardium and differences in the etiologies of ventricular dysfunction in children compared with adults [exist]... invalidating the concept that children can safely be considered small adults for the purpose of understanding heart failure pathophysiology and treatment."[22]

44. In discussing this issue further, Dr. Lipshultz claims, "[t]he disease processes resulting in ventricular dysfunction are often different in children than adults. Many pediatric conditions have no close analogies in the adult...[hence] the effects of intervention may be unlike those seen in adults."[23]

45. Finally, Dr. Lipshultz notes, "when trying to understand the proper therapy for children with ventricular dysfunction it is usually important not to view the child as a small adult and extrapolate the effects of ventricular dysfunction therapy for adult ischemia or post-infarction patients to the child where a multitude of non-ischemic, non post-infarction etiologies exist."[24]

46. Thus, iNO clinical studies involving adults cannot be extrapolated to children, neonates or near-term neonates.

REVIEW OF PRIOR ART CITED IN PENDING OFFICE ACTION

47. *NIH Clinical Center, Department Policy and Procedure Manual for the Critical Care Therapy and Respiratory Care Section; Nitric Oxide Therapy,* 2000. This appears to be an internal policy and procedure directed towards Respiratory Therapists who work in the Medical Intensive Care Unit of the Warren G. Magnuson Clinical Center (the NIH hospital), and are responsible for administering iNO. This policy and procedure states, "NO has been approved for use in the treatment of term or near term (>34 weeks) neonates with hypoxic respiratory failure associated with clinical or

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[21] Steven E. Lipshultz, Ventricular dysfunction clinical research in infants, children and adolescents, *Progress in Pediatric Cardiology*, 12 (2000) 1-28.
[22] *Id.*
[23] *Id.*
[24] *Id.*

echocardiographic evidence of pulmonary hypertension." The manual goes on to note, "[o]btaining NO drug for any other indication than the one specified [treatment of term and near term neonates] is very costly and requires additional administrative actions. [Therefore], in the MICU [medical intensive care unit], NO will be used in the following manner."[25] The manual then lays out the situations in which Respiratory Therapists will be called upon to assist in administering NO, namely, in the support of research protocols and clinical support of inpatients. Presumably, since the Warren G. Magnuson Clinical Center does not have a neonatal intensive care unit,[26] and adult patients are admitted to the 12 bed MICU, this referenced Policy and Procedure Manual would be inapplicable to neonates. Further, the examiner notes the manual's reference to severe left ventricular failure as a contraindication. This is however, a relative contraindication[27] and not an exclusion as noted in the discussion, *supra*, in ¶¶16-28.

48. *Atz, et al., Seminars in Perinatology, 1997*. This article reviewed data collected from the treatment of 400 patients (37% newborns) with iNO. Nearly two-thirds of those patients exhibited pulmonary hypertension associated with congenital heart disease. These patients underwent early surgical repair of their congenital heart defects at Boston's Children's Hospital. When discussing the use of iNO in the newborn with severe LVD and pulmonary hypertension, the authors point out that in these newborns the use of iNO should be with extreme caution, if at all.[28] The authors further noted that these patients had a particularly specific set of anatomic and hemodynamic characteristic where the systemic circulation may depend in part on the ability of the right ventricle to sustain cardiac output via a right-to-left shunt across the patient's patent ductus arteriosus. The authors further cite to other studies reporting adverse outcomes in this circumstance.[29] Hence, even considering the referenced

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[25] *See*, NIH Clinical Center, Department Policy and Procedure Manual for the Critical Care Therapy and Respiratory Care Section; Nitric Oxide Therapy, 2000, §§ 3.1, 3.1.1, 3.1.2. (emphasis added)
[26] *See*, http://www.cc.nih.gov/ccmd/clinical_services.html
[27] *See*, NIH Clinical Center, Department Policy and Procedure Manual for the Critical Care Therapy and Respiratory Care Section; Nitric Oxide Therapy, 2000, § 5.2.3.
[28] Atz, et al., Inhaled Nitric Oxide in the Neonate with Cardiac Disease, Seminars in Perinatology, Volume 21, No. 5 (October), 1997, pp.441-455.
[29] Wessel, et al., Improved Oxygenation in a Randomized Trial of Inhaled Nitric Oxide for Persistent Pulmonary Hypertension of the Newborn, Pediatrics, Vol 100, Number 5, 1997. In this study, the authors randomized 49 mechanically ventilated newborns with severe persistent pulmonary hypertension. Patients with gestational age of less than 34 weeks or with congenital heart disease or diaphragmatic hernia were excluded from the study.

articles to the comment that iNO should be used "with extreme caution, if at all," nowhere does the article suggest that patients with LVD as evidenced by an elevated PCWP or a PCWP>20 mmHg (or other means) should be **excluded** from therapeutic treatment with iNO due to a risk of pulmonary edema. Moreover, the article provides no data to support the author's opinion.

49. *Loh, E., et al., Circulation, 1994.* This is a study of 19 patients with an average age of 52 +/- 3 years. These **adult patients** suffered from ischemic cardiomyapathy (heart failure due to coronary artery disease and resultant partial cardiac muscle death) and idiopathic dilated cardiomyopathy, which resulted in left ventricular failure. These patients also had reactive pulmonary artery HTN secondary to LV failure. These patients were identified as having heart failure due to LV dysfunction as classified by NYHA classifications (class III and class IV). They were not identified as having LV dysfunction as it relates to PCWP or PAWP[30]. The study found that in patients with heart failure due to LV dysfunction, inhalation of NO causes a decrease in the PVR associated with an increase in LV filling pressure. These findings suggest that iNO, if used alone, may have adverse effects in patients with LV failure. There is no mention, however that patients with LV dysfunction as characterized by an elevated PCWP or PAWP should not receive iNO. Finally, this study only identified LVD in **older adults, not neonates**, so it is not relevant to the claimed invention concerning children.[31]

50. *Kinsella, et al, The Lancet, 1999.* This is a double blind study that evaluated 80 premature infants with severe hypoxic respiratory failure. The patients were randomized into two groups, those who received iNO and those who did not. The rate and severity of intracranial hemorrhage, pulmonary hemorrhage, duration of ventilation, and chronic lung disease were further studied in these two groups. The authors noted that potential adverse effects of iNO on platelet adhesion. They also noted attendant risks of intracranial hemorrhage and the severe consequences of prematurity. Hence,

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However, there was no mention of exclusion of newborns diagnosed with pre-existing LVD. One patient in this study later identified with "poor left ventricular function and a right ventricular dependent circulation with echocardiographic evidence of a small left atrium and left atrial hypertension with continuous right to left ductal flow," died during the study. This was after iNO was administered and systemic hypotension resulted, which led to initiation of ECMO and the patient died from an intracranial hemorrhage. This article is silent with respect to children and neonates being excluded from being administered iNO.

[30] Pulmonary Capillary Wedge Pressure (PCWP) and Pulmonary Artery Wedge Pressure (PAWP) are synonymous.

[31] *Supra* at ¶¶42-46.

they only included neonates with the most severe respiratory failure. They concluded that low dose iNO improved oxygenation but did not improve survival in severely hypoxic neonates. Nowhere in the article do the authors suggest that newborns or children with LV failure or dysfunction (neonates with this pathology were not the subject of this study) should be excluded from treatment with iNO.

51. *Bolooki, Textbook, Clinical Application of the Intra-Aortic Balloon Pump*, 1998. This textbook deals with the clinical uses of the intra-aortic balloon pump, which is not indicated for use in neonates, only adults.

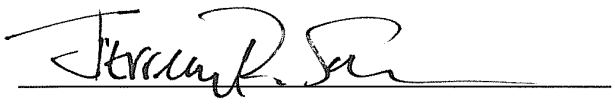
52. *Macrae, et al., Intensive Care Medicine,* 2004. This paper assessed evidence for the use of iNO in the management of neonates and children with cardio respiratory failure and was presented to a consensus meeting jointly organized by the European Society of Pediatric Research and the European Society of Neonatology. Participants in this project were from the UK, France, Germany, Norway, Italy, Sweden, Switzerland, and Spain. The goal was to produce a set of guidelines for the safe use of iNO therapy. In review of medical literature and prior studies, the committee discussed the use of iNO in relation to its dosage, discontinuation and weaning, toxicity, delivery and monitoring, environmental safety, transport, staff training, use in preterm neonates, use in pediatric acute lung injury and acute respiratory distress syndrome, and use in children with cardiac disease. Specifically, under the section "Use of inhaled nitric oxide in children with cardiac disease" the committee is completely silent concerning the issue of exclusionary criteria. Given the quality of experts, the preeminence of the committee, its standing as an authoritative body in Europe, and, the given purpose of this paper to determine guidelines for the use of iNO, it is very significant that there is no mention anywhere of exclusionary criteria relating to LVD, elevated PCWP, or, PCWP>20 mmHg. Thus, It is my opinion that the Committee's silence concerning exclusionary criteria is compelling evidence of the novelty and nonobviousness of the instantly claimed invention.

OTHER PRIOR ART OF RECORD DEMONSTRATING INDICIA OF NONOBVIOUSNESS

53. *Atz, et al., Journal of the American College of Cardiology, 1999.* This is a study of 71 patients with pulmonary hypertension.[32] Prognosis and proper treatment of patients with this condition is dependent, in part, upon pulmonary vasoreactivity. The study compared two groups of patients: one group received NO and O_2 combined, and the other group received NO and O_2 alone. Results indicated that the group receiving combined NO and O_2 were identified as having significant pulmonary vasoreactivity, which might not be the case if O_2 and NO were used separately. Importantly, the article states that in this study "no patient demonstrated clinically important pulmonary edema, hemodynamically significant vasoconstriction or decreased cardiac index during the brief administration of O_2 or NO in O_2."[33] Thus, in my expert medical opinion, this article provides further evidence of indicia of the nonobviousness of the instantly claimed invention.

54. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of any patent issuing from this patent application.

Dated: 30 Sept 2010



Jeffrey R. Smith, Esq.

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[32] Atz, et al., Combined Effects of Nitric Oxide and Oxygen During Acute Pulmonary Vasodilator Testing, Journal of the American College of Cardiology, Vol 33, No 3, (1999).
[33] *Id.* at 818.

Research Subject Resources

Adverse Events Reporting

Adverse events (AEs) may occur during a clinical trial. Current federal regulations require reporting of AEs in all categories of clinical trials to the institutional IRB, in addition to other relevant parties. Other possible destinations for AE reports include the sponsor (if an IND is involved), the FDA (for AEs from commercially available agents), and, if gene transfer is involved, the NIH Office of Biotechnology Activities (OBA).

Definition of Adverse Events

University of California, Irvine
An adverse event (AE) is an unanticipated negative effect. AE reports must be filed with the Institutional Review Board (IRB) when any of the following happen to a subject on a study:

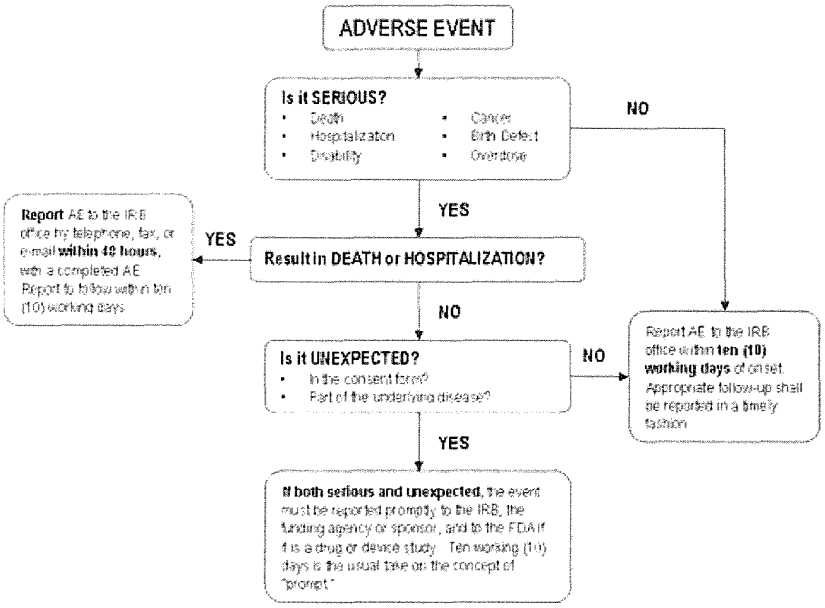
1. Death
2. Hospitalization (including extension of a planned hospital stay)
3. Unanticipated negative effect requiring treatment
4. Unusual or high frequency of expected effects (as described in the "Risks" section of the approved informed consent document)
5. Any other suspicious negative effect when, in the opinion of the Lead Researcher, there may be a relationship to the study
6. Birth defect/congenital anomaly (adverse pregnancy outcome following exposure to study procedures prior to conception or during pregnancy)

Federal Guidelines – Common Rule
The notification requirements described in the Common Rule define adverse events as "unanticipated problems" involving risks to study participants or others.

Federal Guidelines – National Institutes of Health
Generally, the funding Institutes and Centers establish operational definitions of adverse events that apply to the particular trial. The National Cancer Institute (NCI), for example, defines adverse drug reactions in its clinical trials involving antineoplastic agents, as: (1) previously unknown toxicities; and (2) life-threatening or fatal toxicities regardless of whether or not previously unknown. Toxicity criteria are generally included in the protocols.

Federal Guidelines – Food and Drug Administration
The FDA, in Federal regulations 21 CFR Part 312, defines adverse events as any untoward medical occurrence that may present itself during treatment or administration with a pharmaceutical product, and which may or may not have a causal relationship with the treatment. In the guideline entitled "Clinical Safety Data Management: Definitions and Standards for Expedited Reporting", the Agency further clarifies and defines serious adverse events stemming from a drug study as any untoward medical occurrence that at any dose results in death; is life-threatening; requires inpatient hospitalization or prolongation of existing hospitalization; creates persistent or significant disability/incapacity, or a congenital anomaly/birth defects (http://www.fda.gov/cder/guidance/iche3.pdf).

Adverse Event Reporting



Relevant Links

http://www.rgs.uci.edu/researchprotections/irb/adverseexperiences.htm
http://grants.nih.gov/grants/guide/notice-files/not99-107.html
http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=312
http://ohrp.osophs.dhhs.gov/humansubjects/guidance/45cfr46.htm

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Guideline for Industry

Clinical Safety Data Management: Definitions and Standards for Expedited Reporting

ICH-E2A

March 1995

TABLE OF CONTENTS

GUIDELINE FOR INDUSTRY[1]

CLINICAL SAFETY DATA MANAGEMENT:

DEFINITIONS AND STANDARDS FOR EXPEDITED REPORTING[2]

I. INTRODUCTION

It is important to harmonize the way to gather and, if necessary, to take action on important clinical safety information arising during clinical development. Thus, agreed definitions and terminology, as well as procedures, will ensure uniform Good Clinical Practice standards in this area. The initiatives already undertaken for marketed medicines through the CIOMS-1 and CIOMS-2 Working Groups on expedited (alert) reports and periodic safety update reporting, respectively, are important precedents and models. However, there are special circumstances

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[1]This guideline was developed within the Expert Working Group (Efficacy) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at *Step 4* of the ICH process, October 27, 1994. At *Step 4* of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and the USA. This guidance was published in the Federal Register on March 1, 1995 (60 FR 11284) and is applicable to both drug and biological products. In the past, guidelines have generally been issued under § 10.90(b) [21 CFR 10.90(b)], which provides for the use of guidelines to state procedures or standards of general applicability that are not legal requirements but that are acceptable to FDA. The agency is now in the process of revising §10.90(b). Therefore, this guideline is not being issued under the authority of §10.90(b), and it does not create or confer any rights, privileges or benefits for or on any person, nor does it operate to bind FDA in any way. For additional copies of this guideline contact the Executive Secretariat Staff, HFD-8, Center for Drug Evaluation and Research, 7500 Standish Place, Rockville, MD 20855, 301-594-1012. An electronic version of this guideline is also available via Internet by connecting to the CDER FTP server (CDVS2.CDER.FDA.GOV) using the FTP protocol.

[2]The time frames and definitions in this guideline differ from those in the Code of Federal Regulations [21 CFR 314.80]. Until the regulations are revised, the time frames and definitions in the CFR should be followed.

involving medicinal products under development, especially in the early stages and before any marketing experience is available. Conversely, it must be recognized that a medicinal product will be under various stages of development and/or marketing in different countries, and safety data from marketing experience will ordinarily be of interest to regulators in countries where the medicinal product is still under investigational only (Phase 1, 2, or 3) status. For this reason, it is both practical and well-advised to regard premarketing and post-marketing clinical safety reporting concepts and practices as interdependent, while recognizing that responsibility for clinical safety within regulatory bodies and companies may reside with different departments, depending on the status of the product (investigational vs. marketed).

There are two issues within the broad subject of clinical safety data management that are appropriate for harmonization at this time:

- the development of standard definitions and terminology for key aspects of clinical safety reporting, and

- the appropriate mechanism for handling expedited (rapid) reporting, in the investigational (i.e., pre-approval) phase.

The provisions of this guideline should be used in conjunction with other ICH Good Clinical Practice guidelines.

II. DEFINITIONS AND TERMINOLOGY ASSOCIATED WITH CLINICAL SAFETY EXPERIENCE

A. Basic Terms

Definitions for the terms adverse event (or experience), adverse reaction, and unexpected adverse reaction have previously been agreed to by consensus of the more than 30 Collaborating Centers of the WHO International Drug Monitoring Centre (Uppsala, Sweden). [Edwards, I.R., et al, "Harmonisation in Pharmacovigilance," *Drug Safety* 10(2): 93-102, 1994.] Although those definitions can pertain to situations involving clinical investigations, some minor modifications are necessary, especially to accommodate the pre-approval, development environment.

The following definitions, with input from the WHO Collaborative Centre, have been agreed:

1. Adverse Event (or Adverse Experience)

   Any untoward medical occurrence in a patient or clinical

investigation subject administered a pharmaceutical product and which does not necessarily have to have a causal relationship with this treatment.

An adverse event (AE) can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding, for example), symptom, or disease temporally associated with the use of a medicinal product, whether or not considered related to the medicinal product.

2. Adverse Drug Reaction (ADR)

In the *pre-approval clinical experience* with a new medicinal product or its new usages, particularly as the therapeutic dose(s) may not be established:

> all noxious and unintended responses to a medicinal product related to any dose should be considered adverse drug reactions.

The phrase "responses to a medicinal products" means that a causal relationship between a medicinal product and an adverse event is at least a reasonable possibility, i.e., the relationship cannot be ruled out.

Regarding *marketed medicinal products*, a well-accepted definition of an adverse drug reaction in the post-marketing setting is found in WHO Technical Report 498 [1972] and reads as follows:

> A response to a drug which is noxious and unintended and which occurs at doses normally used in man for prophylaxis, diagnosis, or therapy of disease or for modification of physiological function.

The old term "side effect" has been used in various ways in the past, usually to describe negative (unfavorable) effects, but also positive (favorable) effects. It is recommended that this term no longer be used and particularly should not be regarded as synonymous with adverse event or adverse reaction.

3. Unexpected Adverse Drug Reaction

An adverse reaction, the nature or severity of which is not consistent with the applicable product information (e.g.,

Investigator's Brochure for an unapproved investigational medicinal product). See section III.C.

B. Serious Adverse Event or Adverse Drug Reaction

During clinical investigations, adverse events may occur which, if suspected to be medicinal product-related (adverse drug reactions), might be significant enough to lead to important changes in the way the medicinal product is developed (e.g., change in dose, population, needed monitoring, consent forms). This is particularly true for reactions which, in their most severe forms, threaten life or function. Such reactions should be reported promptly to regulators.

Therefore, special medical or administrative criteria are needed to define reactions that, either due to their nature ("serious") or due to the significant, unexpected information they provide, justify expedited reporting.

To ensure no confusion or misunderstanding exist of the difference between the terms "serious" and "severe," which are not synonymous, the following note of clarification is provided:

> The term "severe" is often used to describe the intensity (severity) of a specific event (as in mild, moderate, or severe myocardial infarction); the event itself, however, may be of relatively minor medical significance (such as severe headache). This is *not* the same as "serious," which is based on patient/event *outcome or action* criteria usually associated with events that pose a threat to a patient's life or functioning. Seriousness (not severity) serves as a guide for defining regulatory reporting obligations.

After reviewing the various regulatory and other definitions in use or under discussion elsewhere, the following definition is believed to encompass the spirit and meaning of them all:

> A serious adverse event (experience) or reaction is any untoward medical occurrence that at any dose:
>
> - Results in death,
> - Is life-threatening,
>
> NOTE: The term "life-threatening" in the definition of "serious" refers to an event in which the patient was at risk

of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

- Requires inpatient hospitalization or prolongation of existing hospitalization,
- Results in persistent or significant disability/incapacity, or
- Is a congenital anomaly/birth defect.

Medical and scientific judgment should be exercised in deciding whether expedited reporting is appropriate in other situations, such as important medical events that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the patient or may require intervention to prevent one of the other outcomes listed in the definition above. These should also usually be considered serious.

Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm; blood dyscrasias or convulsions that do not result in hospitalization; or development of drug dependency or drug abuse.

C. Expectedness of an Adverse Drug Reaction

The purpose of expedited reporting is to make regulators, investigators, and other appropriate people aware of new, important information on serious reactions. Therefore, such reporting will generally involve events previously unobserved or undocumented, and a guideline is needed on how to define an event as "unexpected" or "expected" (expected/unexpected from the perspective of previously observed, *not* on the basis of what might be anticipated from the pharmacological properties of a medicinal product).

As stated in the definition (II.A.3.), an "unexpected" adverse reaction is one, the nature or severity of which is not consistent with information in the relevant source document(s). Until source documents are amended, expedited reporting is required for additional occurrences of the reaction.

The following documents or circumstances will be used to determine whether an adverse event/reaction is expected:

1. For a medicinal product not yet approved for marketing in a country, a company's Investigator's Brochure will serve as the

source document in that country. See section III.F. and ICH Guideline for the Investigator's Brochure.

2. Reports which add significant information on specificity or severity of a known, already documented serious ADR constitute unexpected events. For example, an event more specific or more severe than described in the Investigator's Brochure would be considered "unexpected." Specific examples would be (a) acute renal failure as a labeled ADR with a subsequent new report of interstitial nephritis and (b) hepatitis with a first report of fulminant hepatitis.

III. STANDARDS FOR EXPEDITED REPORTING

A. What Should be Reported?

1. Single Cases of Serious, Unexpected ADRs

All ADRs that are both serious and unexpected are subject to expedited reporting. This applies to reports from spontaneous sources and from any type of clinical or epidemiological investigation, independent of design or purpose. It also applies to cases not reported directly to a sponsor or manufacturer (for example, those found in regulatory authority generated ADR registries or in publications). The source of a report (investigation, spontaneous, other) should always be specified.

Expedited reporting of reactions that are serious but *expected* will ordinarily be inappropriate. Expedited reporting is also inappropriate for serious events from clinical investigations that are considered *not* related to study product, whether the event is expected or not. Similarly, nonserious adverse reactions, whether expected or not, will ordinarily not be subject to *expedited* reporting.

Information obtained by a sponsor or manufacturer on serious, unexpected reports from any source should be submitted on an expedited basis to appropriate regulatory authorities if the minimum criteria for expedited reporting can be met. See section III.B.

Causality assessment is required for clinical investigation cases. All cases judged by either the reporting health care professional or the sponsor as having a reasonable suspected causal relationship

to the medicinal product qualify as ADRs. For purposes of reporting, adverse event reports associated with marketed drugs (spontaneous reports) usually imply causality.

Many terms and scales are in use to describe the degree of causality (attributability) between a medicinal product and an event, such as certainly, definitely, probably, possibly or likely related or not related. Phrases such as "plausible relationship," "suspected causality," or "causal relationship cannot be ruled out" are also invoked to describe cause and effect. However, there is currently no standard international nomenclature. The expression "reasonable causal relationship" is meant to convey in general that there are facts (evidence) or arguments to suggest a causal relationship.

2. Other Observations

There are situations in addition to single case reports of "serious" adverse events or reactions that may necessitate rapid communication to regulatory authorities; appropriate medical and scientific judgment should be applied for each situation. In general, information that might materially influence the benefit-risk assessment of a medicinal product or that would be sufficient to consider changes in medicinal product administration or in the overall conduct of a clinical investigation represents such situations. Examples include:

a. For an "expected," serious ADR, an increase in the rate of occurrence which is judged to be clinically important.

b. A significant hazard to the patient population, such as lack of efficacy with a medicinal product used in treating life-threatening disease.

c. A major safety finding from a newly completed animal study (such as carcinogenicity) .

B. Reporting Time Frames

1. Fatal or Life-Threatening Unexpected ADRs

Certain ADRs may be sufficiently alarming so as to require very rapid notification to regulators in countries where the medicinal product or indication, formulation, or population for the medicinal

product are still not approved for marketing, because such reports may lead to consideration of suspension of, or other limitations to, a clinical investigation program. Fatal or life-threatening, unexpected ADRs occurring in clinical investigations qualify for very rapid reporting. Regulatory agencies should be notified (e.g., by telephone, facsimile transmission, or in writing) as soon as possible but no later than 7 calendar days after first knowledge by the sponsor that a case qualifies, followed by as complete a report as possible within 8 additional calendar days. This report should include an assessment of the importance and implication of the findings, including relevant previous experience with the same or similar medicinal products.

2. All Other Serious, Unexpected ADRs

Serious, unexpected reactions (ADRs) that are not fatal or life-threatening must be filed as soon as possible but no later than 15 calendar days after first knowledge by the sponsor that the case meets the minimum criteria for expedited reporting.

3. Minimum Criteria for Reporting

Information for final description and evaluation of a case report may not be available within the required time frames for reporting outlined above. Nevertheless, for regulatory purposes, initial reports should be submitted within the prescribed time as long as the following minimum criteria are met: an identifiable patient; a suspect medicinal product; an identifiable reporting source; and an event or outcome that can be identified as serious and unexpected, and for which, in clinical investigation cases, there is a reasonable suspected causal relationship. Follow-up information should be actively sought and submitted as it becomes available.

C. How to Report

The CIOMS-I form has been a widely accepted standard for expedited adverse event reporting. However, no matter what the form or format used, it is important that certain basic information/data elements, when available, be included with any expedited report, whether in a tabular or narrative presentation. The listing in Attachment 1 addresses those data elements regarded as desirable; if all are not available at the time of expedited reporting, efforts should be made to obtain them. See section III.B.

All reports must be sent to those regulators or other official parties requiring them (as appropriate for the local situation) in countries where the drug is under development.

D. Managing Blinded Therapy Cases

When the sponsor and investigator are blinded to individual patient treatment (as in a double-blind study), the occurrence of a serious event requires a decision on whether to open (break) the code for the specific patient. If the investigator breaks the blind, then it is assumed the sponsor will also know the assigned treatment for that patient. Although it is advantageous to retain the blind for all patients prior to final study analysis, when a serious adverse reaction is judged reportable on an expedited basis, it is recommended that the blind be broken only for that specific patient by the sponsor even if the investigator has not broken the blind. It is also recommended that, when possible and appropriate, the blind be maintained for those persons, such as biometrics personnel, responsible for analysis and interpretation of results at the study's conclusion.

There are several disadvantages to maintaining the blind under the circumstances described which outweigh the advantages. By retaining the blind, placebo and comparator (usually a marketed product) cases are filed unnecessarily. When the blind is eventually opened, which may be many weeks or months after reporting to regulators, it must be ensured that company and regulatory data bases are revised. If the event is serious, new, and possibly related to the medicinal product, then if the Investigator's Brochure is updated, notifying relevant parties of the new information in a blinded fashion is inappropriate and possibly misleading. Moreover, breaking the blind for a single patient usually has little or no significant implications for the conduct of the

clinical investigation or on the analysis of the final clinical investigation data.

However, when a fatal or other "serious" outcome is the primary efficacy endpoint in a clinical investigation, the integrity of the clinical investigation may be compromised if the blind is broken. Under these and similar circumstances, it may be appropriate to reach agreement with regulatory authorities in advance concerning serious events that would be treated as disease-related and not subject to routine expedited reporting.

E. Miscellaneous Issues

1. Reactions Associated with Active Comparator or Placebo Treatment

   It is the sponsor's responsibility to decide whether active comparator drug reactions should be reported to the other manufacturer and/or directly to appropriate regulatory agencies. Sponsors should report such events to either the manufacturer of the active control or to appropriate regulatory agencies. Events associated with placebo will usually not satisfy the criteria for an ADR and, therefore, for expedited reporting.

2. Products with More Than One Presentation or Use

   To avoid ambiguities and uncertainties, an ADR that qualifies for expedited reporting with one presentation of a product (e.g., a dosage form, formulation, delivery system) or product use (e.g., for an indication or population), should be reported or referenced to regulatory filings across other product presentations and uses.

   It is not uncommon that more than one dosage form, formulation, or delivery system (oral, IM, IV, topical, etc.) of the pharmacologically active compound(s) is under study or marketed; for these different presentations there may be some marked differences in the clinical safety profile. The same may apply for a given product used in different indications or populations (single dose vs. chronic administration, for example). Thus, "expectedness" may be product or product use specific, and separate Investigator's Brochures may be used accordingly. However, such documents are expected to cover ADR information that applies to all affected product presentations and uses. When relevant, separate discussions of pertinent product-specific or use-specific safety information will also be included.

   It is recommended that any adverse drug reactions that qualify for expedited reporting observed with one product dosage form or use be cross referenced to regulatory records for all other dosage forms and uses for that product. This may result in a certain amount of overreporting or unnecessary reporting in obvious situations (for example, a report of phlebitis on IV injection sent to authorities in a country where only an oral dosage form is studied or marketed). However, underreporting is completely avoided.

3. Post-study Events

Although such information is not routinely sought or collected by the sponsor, serious adverse events that occurred after the patient had completed a clinical study (including any protocol required post-treatment follow-up) will possibly be reported by an investigator to the sponsor. Such cases should be regarded for expedited reporting purposes as though they were study reports. Therefore, a causality assessment and determination of expectedness are needed for a decision on whether or not expedited reporting is required.

F. Informing Investigators and Ethics Committees/Institutional Review Boards of New Safety Information

International standards regarding such communication are discussed within the ICH GCP Guidelines, including the addendum on "Guideline for the Investigator's Brochure." In general, the sponsor of a study should amend the Investigator's Brochure as needed, and in accord with any local regulatory requirements, so as to keep the description of safety information updated.

IV. REFERENCE

Federal Register. Vol.60, No. 40, Wednesday, March 1, 1995, pages 11284-11287.

Attachment 1

KEY DATA ELEMENTS FOR INCLUSION IN EXPEDITED REPORTS OF SERIOUS ADVERSE DRUG REACTIONS

The following list of items has its foundation in several established precedents, including those of CIOMS-I, the WHO International Drug Monitoring Centre, and various regulatory authority forms and guidelines. Some items may not be relevant depending on the circumstances. The minimum information required for expedited reporting purposes is: an identifiable patient, the name of a suspect medicinal product, an identifiable reporting source, and an event or outcome that can be identified as serious and unexpected and for which, in clinical investigation cases, there is a reasonable suspected causal relationship. Attempts should be made to obtain follow-up information on as many other listed items pertinent to the case.

1. **Patient Details:**

   - Initials,
   - Other relevant identifier (clinical investigation number, for example),
   - Gender,
   - Age and/or date of birth,
   - Weight,
   - Height,

2. **Suspected Medicinal Product(s)**:

   - Brand name as reported,
   - International Non-Proprietary Name (INN),
   - Batch number,
   - Indication(s) for which suspect medicinal product was prescribed or tested,
   - Dosage form and strength,

- Daily dose and regimen (specify units - e.g., mg, mL, mg/kg),
- Route of administration,
- Starting date and time of day,
- Stopping date and time, or duration of treatment.

3. Other Treatment(s):

- For concomitant medicinal products (including non-prescription/OTC medicinal products) and non-medicinal product therapies, provide the same information as for the suspected product.

4. Details of Suspected Adverse Drug Reaction(s):

- Full description of reaction(s) including body site and severity, as well as the criterion (or criteria) for regarding the report as serious should be given. In addition to a description of the reported signs and symptoms, whenever possible, attempts should be made to establish a specific diagnosis for the reaction.
- Start date (and time) of onset of reaction,
- Stop date (and time) or duration of reaction,
- Dechallenge and rechallenge information,
- Setting (e.g., hospital, out-patient clinic, home, nursing home),
- Outcome: Information on recovery and any sequelae; what specific tests and/or treatment may have been required and their results; for a fatal outcome, cause of death and a comment on its possible relationship to the suspected reaction should be provided. Any autopsy or other post-mortem findings (including a coroner's report) should also be provided when available. Other information: anything relevant to facilitate assessment of the case, such as medical history including allergy, drug or alcohol abuse; family history; findings from special investigations.

5. Details on Reporter of Event (Suspected ADR):

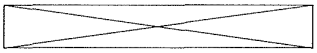
- Name,
- Address,
- Telephone number,
- Profession (speciality).

6. **Administrative and Sponsor/Company Details**:

- Source of report: Was it spontaneous, from a clinical investigation (provide details), from the literature (provide copy), other?
- Date event report was first received by sponsor/manufacturer,
- Country in which event occurred,
- Type of report filed to authorities: initial or follow-up (first, second, etc.),
- Name and address of sponsor/manufacturer/company,
- Name, address, telephone number, and FAX number of contact person in reporting company or institution,
- Identifying regulatory code or number for marketing authorization dossier or clinical investigation process for the suspected product (for example IND or CTX number, NDA number),
- Sponsor/manufacturer's identification number for the case (This number should be the same for the initial and follow-up reports on the same case).

Dictionary

exclusion



ex·clu·sion [ik skloʻozh'n]
(*plural* ex·clu·sions)

noun

Definition:

1. excluding: the act of excluding something or somebody

2. being excluded: the state of being excluded, especially from mainstream society and its advantages
- *addressing the issue of social exclusion*

3. excluded person or thing: somebody or something that has been excluded

[15th century. < Latin *exclusion-*< *exclus-*, past

exclusive economic zone

exclusive listing

participle of *excludere* (see exclude)]

- **ex·clu·sion·ar·y** *adjective*

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precaution

precaution

pre·cau·tion [prə káwsh'n] (*plural* pre·cau·tions)

noun

Definition:

1. protection against possible undesirable event: an action taken to protect against possible harm or trouble or to limit the damage if something goes wrong

2. caution to forestall future trouble: the foresight to protect against possible harm or trouble or to limit the damage if something goes wrong

[Late 16th century. Via French< late Latin *precaution*-< Latin *precaut*-, past participle of *praecavere*, literally "take care before" < *cavere* "take heed"]

- **pre·cau·tion·al** *adjective*
- **pre·cau·tion·ar·y** *adjective*
- **pre·cau·tious** *adjective*

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DERMATOLOGICAL CRYOSURGERY IN PRIMARY CARE WITH DIMETHYL ETHER PROPANE SPRAY IN COMPARISON WITH LIQUID NITROGEN

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CUTANEOUS CRYOSURGERY IN FAMILY MEDICINE: DIMETHYL ETHER PROPANE SPRAY VERSUS LIQUID NITROGEN

Objective. To compare the efficacy, tolerance and safety of two types of cryotherapy, performed by family physicians, for benign cutaneous lesions: low freezing (-57°C) with dimethyl ether propane cryogenic spray (DMEP) and intense freezing (-196°C) with conventional liquid nitrogen (LN).

Design. A randomized, multi-centered, controlled clinical trial, with single-blind assessment.

Setting. Three primary care teaching teams in the Community of Madrid.

Patients and other participants. Ten MIR from family & community medicine intervened. There were 124 patients, who had 174 benign cutaneous lesions, suitable for cryotherapy. There were 3 voluntary withdrawals, none because of an adverse reaction.

Interventions. In each case there was local application for a standard time of the randomized agent. Control-group intervention, 81 cases: swab soaked in LN. Study-group intervention, 93 cases: swab saturated with DMEP spray. Maximum of three freezings per case, at weekly intervals.

Measurements and main results. A doctor made a blind assessment of the results (elimination, adverse reaction, aesthetic result) 15 days after treatment.

Conclusions. No clinically relevant differences between the efficacy, tolerance and safety of the two cryogenic agents used in primary care were found. The low freezing of DMEP was sufficient for the cryotherapy of benign lesions.

1. INTRODUCTION

Dermatological cryosurgery enables destruction of a wide variety of superficial skin lesions by controlled freezing. Because of its safety and high level of effectiveness (1-4) and because it is easy to learn to use the method, it is widely used in Anglo-Saxon countries by doctors who are not dermatological specialists (5-7).

The simplest method of freezing is topical application on lesions which one wishes to destroy of a cottonwool swab saturated by immersion in liquid nitrogen (LN). This cryogenic agent, having a temperature of -196°C, is very effective in elimination of a large variety of very common benign and premalignant skin lesions (verrucas, molluscum contagiosum, seborrheic and actinic keratoses...). Unfortunately, because of its extremely low boiling point, the substance has to be stored in special containers which are not available in the Health Centers in our environment. An infrastructural deficiency is therefore the main limiting factor for cryosurgery in general medical practice in Spain.

In our Teaching Unit, a regular supply of small quantities of the cryogenic agent in portable, domestic type thermos flasks from the reference Dermatological Department (Puerta de Hierro Hospital) has enabled us to carry out cryosurgery with liquid nitrogen for the past few years, as a routine method and with good results. Because of the rapid evaporation of the product transported in this way, it is essential to use it within a few hours of receipt of the same. In order to make the method cost-effective, therefore, it is necessary to gather together patients to be treated on the days on which one will be receiving the product.

Since the treatment is excessively dependent on the willingness of participants, this experience is still an exceptional situation in Primary Care in this field of medicine. In fact, in June 1994, only 0.8% of tutors and third year resident general practitioners of the 26 Primary Care Teams of the Community of Madrid were regularly practicing cryosurgery, the usual practice being to use less decisive alternatives (keratolytics) or, unnecessarily, to refer patients to busy departments specializing in minor pathology.

A coolant mixture of dimethyl ether and propane (DMEP) has recently been marketed in aerosol form, which is easy to administer and also to store; its small container makes it easy to transport and keeps it stable for three years with no special precautions. Through evaporation this product reaches -57°C in its applicator swab. Our theory was that if this temperature, which is markedly higher than that obtained with liquid nitrogen, was found to be adequate for destruction of skin lesions, this kit would represent an answer to logistic problems standing in the way of practicing cryosurgery in the consulting rooms of general practitioners.

The bibliography available to date on DMEP spray (11) describes some small trials using the product without control groups; the real effectiveness of this low freezing cryosurgery is therefore as yet unknown. In this study, we present the results of the

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first clinical trial of the product in comparison with standard cryotherapy with liquid nitrogen in elimination of benign skin lesions, for the purpose of providing the doctor with scientific criteria on the basis of which to assess the advantage of the new therapeutic alternative in his daily practice.

2. MATERIAL AND METHOD

Design of the Study. A controlled, randomized, parallel experimental trial, with blind assessment of the main result, to compare the effectiveness of DMEP in elimination of benign skin lesions with standard cryotherapy (LN). Tolerance and safety of both systems are analyzed secondarily. The trial was designed and monitored in the Family and Community Medicine Teaching Unit of Madrid Area 6, with the mandatory approval of the Clinical Research Ethics Committee of Puerta de Hierro Hospital.

Scope and Period of the Trial. The field work was carried out in the clinics of three Primary Care Teams of the Teaching Unit (Majadahonda PCT [=Primary Care Team], Arguelles PCT and Pozuelo de Alarcón PCT) by 10 third year house physicians assigned to the said centers during 1995, supervised by their respective tutors, between June and October 1995. All these doctors had prior experience of conventional cryosurgery with LN.

Selection of Study Subjects. Out of the complete range of benign skin lesions suitable for cryotherapy diagnosed in the clinics during the period of the trial, the following 5 complaints were accepted as study subjects, after separate confirmation of the diagnosis of two researchers: verruca plana, verruca vulgaris, verruca filiformis, molluscum contagiosum and seborrheic keratoses. Recruitment of cases continued until a minimum of 80 lesions per treatment group had been obtained, representing a sample of adequate size to enable detection of a difference equal to or greater than 15% between the percentage of lesions eliminated by each agent (95% cures expected with LN, assuming a bilateral contrast having a level of significance of 0.05 and a study power of 0.80). Absence of the exclusion criteria stated in Table 1 was confirmed in each case, and each patient's specific consent was requested after they had received oral and written information.

Procedures Compared. Freezing was carried out by contacting the skin lesions with identical cottonwool swabs (the swabs supplied in the DMEP kit) saturated in the cryogenic products by immersion for a minimum of 10 seconds in LN (reference procedures) or by spraying with the DMEP spray in accordance with the manufacturer's specifications (index procedures). Freezing times (swab-skin contact) were standardized in accordance with standard recommendations in the bibliography for each type of lesion: 20 seconds for verruca plana and molluscum contagiosum, 40 seconds for verruca vulgaris, verruca filiformis and seborrheic keratoses, ensuring in each case that a perilesional halo of healthy skin measuring from 1 to 3 mm was covered. In the event of incomplete elimination of the lesion, repetition was permitted up to a maximum of three freezings (or until such time as the cure enabled assessment of the need for retreatment).

Allocation of Procedural Methods. Allocation of treatment according to centers was stratified in such a way that each PCT had a single list of randomized treatments allocated to it correlative to the cases as included in the trial. It was permitted for one and the same patient to contribute up to a maximum of three different lesions to the trial, treated simultaneously or one after the other. In this situation each lesion was considered as one case, receiving its randomized treatment according to a correlative cranio-caudal order of anatomical location.

Trial Variables and Assessment Criteria. Clinical assessment of the patient was carried out at the time of inclusion in the trial, with recording of the characteristics of the skin lesion (diagnosis, size, location, number) and the characteristics of the carrier patient (sex, age, concurrent cutaneous pathology, previous treatments) which might influence the treatment result. Follow-up of the cases was carried out, as a minimum, one week after each freezing and during an extra end-of-trial appointment 15 days after the last application.

A cure was considered obtained if a lesion was eliminated after freezing, i.e., if no vital skin findings compatible with the original skin lesion were detected, even though after-effects of the therapy applied still persisted (necrotic residues of ampullae, epidermal denudation, depigmentation or other changes of coloration, cicatricial tissue). This judgment was made by the blind method in each case by a researcher other than the physician who had performed the therapy, the patient's group being unknown to the assessing

Table 1. **Criteria for Exclusion of Study Cases**

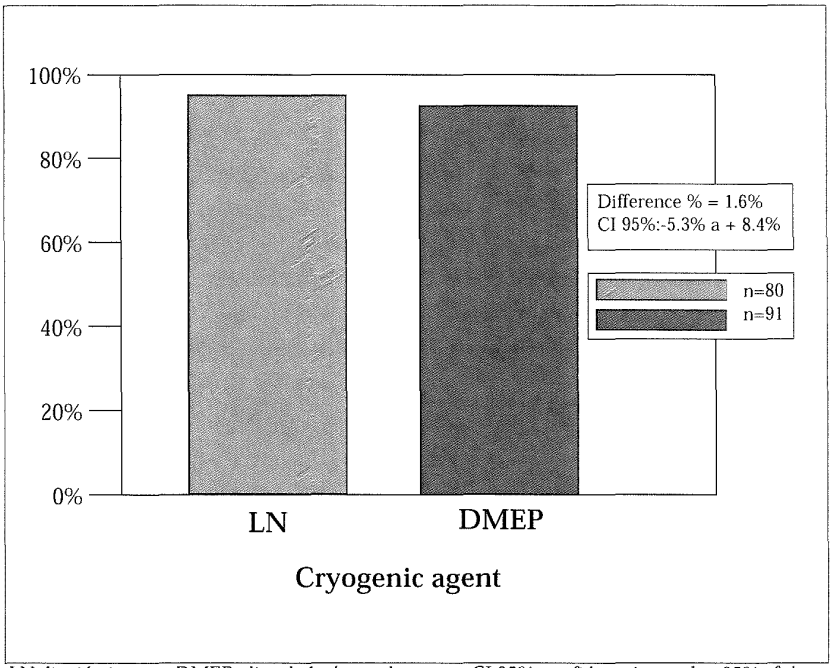
1. -Criteria relating to the site of the lesion
-Area with active skin infection -Areas of potential aesthetic (face) or functional risk (lateral surface of the fingers) -Plantar and genital verrucas
2. -Criteria relating to the diagnosis of the lesion
-Doubtful or discrepant diagnosis in the opinion of two assessing doctors -Pigmented lesions (except for seborrheic keratoses)
3. -Criteria relating to patient's circumstances
-Age less than 6 years or greater than 85 years -Clinical or pathological circumstances which in the operator's opinion render cryotherapy inadvisable (cutaneous or generally important disorder, rough vasculopathic area, cryoagglutinins, terminal patient...) -Other previous, recent treatment of the lesion (in the past 15 days) -Significant adverse effect after other previous cryotherapy

Table 2. **Comparability of the Study Groups**

Characteristics	Liquid Nitrogen (n=80)	Dimethyl Ether Propane (n=91)	p [1]
Characteristics of the patient			
- Sex (male)	52.5%	53.8%	0.86
- Age (average/SD)	24 (15.2)	32.1 (17.3)	<0.01 [2]
Characteristics of the lesion			
- Size (average/SD)	3.9 (3.4)mm	3.2 (1.6)mm	0.54 [2]
- Single lesion	18.7%	17.5%	0.84
- Clinical diagnosis			
Verruca vulgaris	61.2%	56%	0.49
Verruca plana	20%	31.8%	0.07
Molluscum contagiosum	11%	1%	<0.01 [3]
Verruca filiformis	3.7%	6.5%	0.31 [3]
Seborrheic keratoses	3.7%	4.3%	0.57 [3]
- Site			
Head and neck	10%	9.9%	0.98
Upper limbs	61.2%	70.3%	0.21
Lower limbs	11.2%	8.7%	0.59
Trunk	17.5%	14.2%	0.56

SD: standard deviation in mm. (1) p value in chi-square test. (2) p value in Mann-Whitney U test. (3) p value in Fisher's exact test.

Figure 1. **Effectiveness of each cryogenic agent**



LN: liquid nitrogen. DMEP: dimethyl ether and propane. CI 95%: confidence interval at 95% of the difference in percentages. n: lesions treated with each cryogenic agent.

researcher. The aesthetic result was likewise assessed by this dichomotized method at the end of the trial (satisfactory or unsatisfactory). The number of freezings necessary to eliminate the lesion was quantified in each case.

Tolerance of cryotherapy was assessed by asking the patient to describe discomfort perceived during the freezing (none, paresthesia, pruritis, smarting, pain, etc.) quantified in intensity according to a typical scale (none, bearable, treatment interrupted because of discomfort). Safety of the treatment was assessed by recording adverse effects occurring during freezings, identified by questioning the patient and physical examination of the area treated.

In each also the total duration of treatment time was recorded (days elapsing from the start of treatment until restoration of normal skin continuity).

Statistical Analysis. The chi-square test was used for comparison of the proportions of qualitative variables (or Fisher's exact test in the necessary cases). Where necessary, the 95% confidence interval (CI 95%) of the difference of the said percentages was estimated. Mean values of quantitative variables were compared by the Mann-Whitney U test. In all the tests of hypothesis a 95% level of significance was used. Absence of factors of confusion in effectiveness obtained was explored for both types of cryotherapy by means of an unconditional logistical regression model, with the possible modifiers of effectiveness (the aforementioned characteristics of lesion and patient) and the product used taken as independent variables, and elimination (yes/no) of lesions taken as a dependent variable. The computer programs EPIINFO 6.02, SAS for Windows and ENE 2.2 were used for determination of the sample size.

3. RESULTS

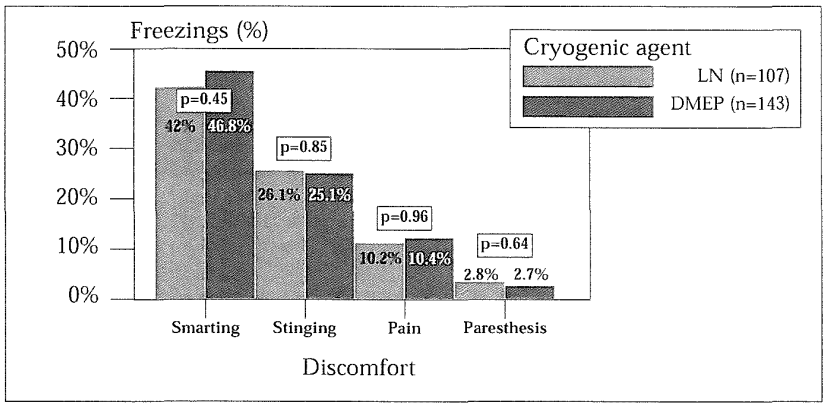
Of the skin lesions potentially suitable for treatment with cryotherapy attended to during the study period, for the exclusion reasons specified (Table 1), 15 cases were not included in the study, 2 refusals to participate being recorded.

Table 3. **Result of each cryogenic agent according to lesion treated**

Lesions	Cases treated with LN %		Cases treated with DMEP %	
	Successes	Failures	Successes	Failures
Verruca vulgaris	46 (94%)	3	48 (94%)	3
Verruca plana	16 (100%)	0	27 (93%)	2
Molluscum contagiosum	9 (100%)	0	1 (100%)	0
Verruca filiformis	3 (100%)	0	5 (83%)	1
Seborrheic keratoses	2 (66%)	1	4 (100%)	0
Total	76 (95%)	4	85 (93%)	6

LN: liquid nitrogen, DMEP: dimethyl ether and propane spray.
Success: lesion eliminated, Failure: lesion persistent after three freezings.

Figure 2. **Characteristics of types of discomfort perceived during freezings**



LN: liquid nitrogen. DMEP: dimethyl ether and propane spray. n: number of freezings with each cryogenic agent. p: value in Fisher's exact test. The figures printed in the bars above represent, for each agent, the % of freezings in which a patient presented the discomfort stated.

Treatment of 174 lesions as study cases was initiated, finally ending with a follow-up of 171 lesions (91 treated with DMEP spray, 80 with LN). The 3 abandoned treatments (two with DMEP, one with LN) were discontinued because it was not possible for the patient to complete the trial protocol [program]. In no case did withdrawal occur for the expected reasons for withdrawal (serious adverse effect or at the patient's express request).

Comparability of the groups resulting after the randomized allocation of treatments was confirmed by univariant analysis of the distribution of characteristics of lesion and carrier patient in each group as summarized in Table 2, no differences of any interest being found. Nor were any significant differences detected in the number of cases treated by each operating physician.

After the complete treatment in accordance with the protocol, 85 lesions treated with DMEP (93.4% of the cases treated) had been eliminated as compared with 76 lesions treated with LN (95% of the cases treated). Contrast of these values by Fisher's exact test gives a p=0.75. Figure 1 gives a graphic representation of the difference between these effectiveness percentages and their corresponding confidence interval. Table 3 summarizes the results obtained by each cryogenic agent on the different types of lesions in the study.

For successfully treated cases an average of 1.26 freezings per lesion destroyed with LN were found in comparison with 1.48 freezings with DMEP (Mann-Whitney U test, p=0.06). Comparison of the distribution of lesions cured by one, two and three freezings with each cryogenic agent (57.18 and 1 with LN, as against 54.21 and 10 with DMEP) showed no differences between the two with a chi-square test with two degrees of freedom corrected by continuity (p>0.05).

In 81.3% of the 107 freezings applied with LN, the patient perceived some discomfort, as compared with 85.33% of the 143 DMEP applications (Fisher's exact test, p=0.39). The CI 95% of the difference found (4%) fluctuated between -5.4% and +13.4%. In no case did the intensity of discomfort prevent completion of the therapy allocated. Figure 2 summarizes the distribution of the types of discomfort perceived by the patient in a comparison of both treatment groups.

Table 4 gives a summary of the 5 cases of minor adverse effects recorded. All cases were healed in a few days of conservative treatment. Of the total number of freezings carried out with each cryogenic agent the complications described represent 1.3% of the DMEP applications as compared with 2.8% of the LN applications. The difference between these percentages (1.5%) lies within the CI 95% range from -2.2% to +5%.

The average time spent on cryotherapy of a skin lesion by each method was 10.2 days with LN and 10.3 days with DMEP (Mann-Whitney U test, p=0.49).

Adjusted by all the variables which could act as possible modifiers of the cryosurgical therapy result (type, size and location of the lesions; age and sex of the patient), by means of an unconditional logistical regression model, non-dependence of the cases of therapy failure on the cryogenic agent applied was confirmed.

4. DISCUSSION

The hypothesis investigated that skin cryosurgery with DMEP spray can be as effective as conventional cryotherapy with liquid nitrogen (LN) cannot be rejected in the light of the results obtained. The differences in percentages of skin lesions cured with one or the other method are neither statistically significant nor clinically relevant.

In consideration of the methodological precautions specified in the design stage, the possibilities of systematic error in the study are low. Firstly, the randomized allocation of the treatments, the homogeneity of the resultant groups and the minimal losses of patients rule out the possibility of gross errors of selection bias. Secondly, and even though it was not possible to hide the treatments from the patients or the operating physicians, the main result of the study (whether the lesion was eliminated or not) can certainly be considered to be blind, since the treatment applied was not revealed to the assessing physician (who was not the operating physician). In this way, the possibilities of information bias were minimized. Similarly, because of their previous experience in conventional cryosurgery, the clinical judgment of the researchers was considered sufficiently capable and specific for measurement of the said main result. In addition to verifying total agreement (100% agreement) between observers in assessment of the results of a pilot sample of 25 lesions having received cryo-treatment, the precaution was taken of reassessing each study case in a final appointment 15 days after application of the final freezing.

Furthermore, the calculations of sample size made beforehand were carried out on the basis of a bibliographic hypothesis of expected results in the control group which totally corresponded to the results obtained in our study. In this way the precautionary measures for sufficient power in the study to detect differences judged as clinically relevant were confirmed.

For all the above reasons, we consider our study to be a true negative result which has not detected differences in the cures achieved by the two methods tested. In both cases over 90% of the skin lesions treated were eliminated, a figure similar to the results obtained with LN by other authors. The temperature reached by the new DMEP spray (low freezing cryosurgery) appears adequate for effective cutaneous destruction of the complaints treated.

Nor did we detect differences in the secondary comparisons of the study, either with regard to discomfort produced in the patient by the one or the other method, or with regard to the adverse effects which occurred. Leaving aside considerations of sample size (which was calculated for the main objective of the study), the good tolerance formerly known for LN is confirmed for DMEP; even though it is usual to feel smarting during the technique, it is perfectly bearable for the majority of patients. It is nevertheless necessary to remember that the freezing of certain areas of the body can be particularly painful (fingernails and toenails, lips, eyelids...). The extremely few complications which occurred were slight and healed spontaneously.

Given these results, the safety of cryotherapy appears manifest. Despite this, a new clinician must guarantee adequate knowledge of the method, as well as of the necessary basic precautions and the few contraindications for the treatment before commencing to practice the same. Various of the bibliographic references of this article are perfectly adequate for these purposes. Even more important than the above-mentioned technical capabilities of execution, which can be acquired by any professional person, is reliability of the doctor's diagnosis, in order to guarantee certain diagnosis of the skin lesion before freezing it. Adequate further training and nearby availability of advice of a dermatologist should prevent destruction of lesions for which histological examination is necessary to enable correct clinical management.

Together with the above-mentioned precautions, even in optimum circumstances of mutual doctor-patient trust, the necessity to obtain the patient's formal consent to the cryosurgery should not be forgotten. This is a legal precaution of a universal nature for any procedure in which clinical risks different from the conventional risks of daily practice can be assumed. Amply distributed printed forms can be used for this.

Table 4. **Adverse effects occurring after 107 freezings with LN and 143 freezings with DMEP.**

Case	Group	Skin Lesions	Complication	Action
1	LN	Seborrheic keratoses	Local infection	Local antiseptic
2	LN	Verruca vulgaris	Hypersensitivity in area treated observation (7 days) (*)	Kept under observation
3	LN	Verruca vulgaris	Hypersensitivity in area treated (5 days) (*)	Kept under observation
4	LN	Verruca plana	Local infection	Local antiseptic
5	DMEP	Verruca vulgaris	Inflammation (superimposed traumatism)	Local antiseptic

LN: liquid nitrogen, DMEP: dimethyl ether and propane spray, (*) healed spontaneously within the period stated.

If we take into consideration, together with all their limitations, the cases not included in the study and the refusals to participate as a sure way of exploring the feasibility of cryosurgery in primary care and the acceptability of patients for this practice by their family doctor, the results discussed would appear to confirm its nature as a suitable method for carrying out in family medicine and as a method which would be well received by patients. This being so, DMEP would provide an answer to a care requirement which is at present not well covered because of lack of infra-structure in Primary Care for handling LN. The DMEP spray kit provides all the necessary equipment for cryosurgery, whilst being small in size and available at a reasonable cost. Also because of its portability, it has enabled us to treat immobilized patients at home during visits to their homes. Combined with good clinical results, we have obtained excellent aesthetic results in all patients, and healing of our cases with a rapidity comparable to that obtained with LN.

These clinical results should be completed by future analyses concerning cost-efficiency between both cryogenic methods. In this way, Anglo-Saxon authors with experience by using the DMEP spray consider it, because of its low infra-structure cost, as the most efficient cryogenic potential in general medical practice.

On the other hand, after our experience with DMEP, together with its obvious advantages we have found a certain disadvantage: the type of ready-made swab fitted on the spray kit, which is supplied in a single, 5mm diameter size, is too big for freezing the smallest lesions. Although the appropriate cryosurgical technique requires inclusion of a perilesional halo in the area to be frozen, this problem which we have encountered can be a source of certain amounts of discomfort which would be avoidable with more accurately sized swabs. This situation has now been rectified by the manufacturer of the product by distribution of different types of applicators.

It must also be pointed out that this study has included exclusively 5 types of specific benign skin lesions, probably those with the highest morbidity rate of the pathologies treatable with cryotherapy in primary care. Until it is irrefutably confirmed, it would not be scientifically permissible to extend the indications of DMEP spray to other types of lesions apart from those referred to here, and especially to premalignant conditions (actinic keratoses, Bowen's disease, ...) which are routinely treated with LN. In experimental cryosurgery a different destruction temperature has been found for normal, dysplastic and cancerous skin cells. As a new research prospect derived from this trial, we shall in the near future study the subject of clinical translation of these data, by means of a new trial with DMEP, to other, different skin lesions.

Likewise, our results can definitely not be extrapolated to other cryogenic sprays of different formulation and physical properties which have not been tested in controlled form for skin freezing: ethyl chloride, Verruca Freeze (not available in our country), etc.

Finally, and in order to provide information which is complementary to this study, we expect to be in a position in a few months' time to provide an analysis of the possible differences of the long term result (rate of relapses) between the two cryogenic agents studied.

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Sibutramine-metformin Combination Versus Sibutramine and Metformin Monotherapy in Obese Patients

This study is ongoing, but not recruiting participants.

First Received: July 14, 2009 Last Updated: July 15, 2009 History of Changes

Sponsor:	Laboratorios Silanes S.A. de C.V.
Information provided by:	Laboratorios Silanes S.A. de C.V.
ClinicalTrials.gov Identifier:	NCT00941382

Purpose

The aim of this study is to evaluate the effect of sibutramine and metformin combination therapy in comparison with sibutramine or metformin monotherapy over weight, adiposity, glucose metabolism and inflammatory state in obese patients.

Condition	Intervention	Phase
Obesity	Drug: Sibutramine-Metformin Drug: Sibutramine Drug: Metformin	Phase III

Study Type: Interventional
Study Design: Allocation: Randomized
Endpoint Classification: Safety/Efficacy Study
Intervention Model: Parallel Assignment
Masking: Double Blind (Subject, Investigator, Outcomes Assessor)
Primary Purpose: Treatment

Official Title: Double-blind, Randomized Clinical Trial to Evaluate Effect of Combination Therapy of Metformin and Sibutramine Versus Metformin or Sibutramine Monotherapy Over Weight, Adiposity, Glucose Metabolism and Inflammatory State in Obese Patients

Resource links provided by NLM:

MedlinePlus related topics: Diabetes Medicines Obesity

Drug Information available for: Sibutramine Sibutramine hydrochloride monohydrate Metformin Metformin hydrochloride

U.S. FDA Resources

Further study details as provided by Laboratorios Silanes S.A. de C.V.:

Primary Outcome Measures:

- improvement of body weight, adiposity and inflammation state defined by serum adiponectin, leptin and C reactive protein [Time Frame: 6 months]
  [Designated as safety issue: No]

Secondary Outcome Measures:

- improvement of metabolic profile, defined by triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, insulin, and insulin sensitivity [Time Frame: 6 months] [Designated as safety issue: No]
- adverse events [Time Frame: 6 months] [Designated as safety issue: Yes]

Estimated Enrollment:	60
Study Start Date:	November 2008
Estimated Study Completion Date:	September 2009
Estimated Primary Completion Date:	August 2009 (Final data collection date for primary outcome measure)

Arms	Assigned Interventions
Sibutramin-Metformin: Experimental Sibutramine-metformin therapy in a single tablet	Drug: Sibutramine-Metformin sibutramine and metformin, 15 mg per day and 850 mg per day, respectively, in a single tablet, for 180 days
Sibutramine: Active Comparator Sibutramine monotherapy	Drug: Sibutramine 15 mg per day for 180 days
Metformin: Active Comparator Metformin monotherapy	Drug: Metformin Metformin 850 mg per day for 180 days

Detailed Description:

The treatment of obesity is strongly recommended because it exacerbates insulin resistance, hypertension, dyslipidemia and atherosclerosis, and represents a risk factor for type 2 diabetes. Although diet and exercise are valuable in this treatment, patient compliance is a major problem. Sibutramine has been shown to be a highly effective pharmacotherapy for weigh loss in obese patients, mediated by increased satiety and an enhancement of energy expenditure. Metformin is widely used for glycemia control and is associated with a small to moderate body weight loss. We are assessing the combination of sibutramine and metformin, two agents with different mechanisms of action for control of body weight and metabolic dysregulation.

Eligibility

Ages Eligible for Study:	30 Years to 50 Years
Genders Eligible for Study:	Both
Accepts Healthy Volunteers:	No

Criteria

Inclusion criteria

- Age between 30 and 50 years
- Both genders
- BMI between 30 and 40
- Stable body weigh defined by over 5 per cent variability during the previous 3 months
- Fasting serum glucose less than 126 mg per dl
- Blood pressure over 140 and 90 mmHg
- Women ensuring contraceptive precautions.
- Communication and understanding capability.
- Informed consent awarding.

Exclusion criteria

- Women were excluded if they were pregnant or lactating potential while no taking adequate contraceptive precautions
- Any smoking during the preceding 6 months
- No physical activity, defined by less than 15 minutes per day of walking
- Excessive physical activity equivalent to running over 60 minutes per day
- Known hypersensitivity to sibutramine or metformin
- Low commitment to follow the protocol statements
- Any investigational medication during the preceding 6 months
- Any drug or substance mayor toxicity exposure during the preceding 3 months
- Alcohol or any drug abuse during the previous 3 months
- Current medication of oral corticosteroids, anticoagulants, sympathomimetics, sympatholytics, lipid lowering drugs, any medication for type 2 diabetes, and any sibutramine interaction drug
- Current or previous evidence of ischemic heart disease, cardiac arrhythmia, cerebrovascular disease, chronic hepatic disease, two fold persistent elevation of ALT, AST or FA
- Carrying a pacemaker or any permanent bioelectronic component that could interfere with bioimpedance process
- Renal failure defined by serum creatinine equal or ever 1.2 mg per dL
- Not controlled thyroid disease defined by altered serum T3, T4 and TSH during the previous 3 months
- Hypertension
- Type 2 diabetes
- Anti-depressants, or any psychiatric disturbance treatment

Contacts and Locations

Please refer to this study by its ClinicalTrials.gov identifier: NCT00941382

Locations

Mexico, Jalisco

Centro Universitario de Ciencias de la Salud
Guadalajara, Jalisco, Mexico, 44340

Sponsors and Collaborators

Laboratorios Silanes S.A. de C.V.

Investigators

Study Director:	Jorge A González-Canudas, MD	Laboratorios Silanes
Study Chair:	Manuel González-Ortiz, PHD	Universidad de Guadalajara
Principal Investigator:	ESperanza Martínez-Abundis, PHD	Universidad de Guadalajara

More Information

Publications:

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Responsible Party:	Laboratorios Silanes S.A. de C.V. (Jorge González Canudas)
ClinicalTrials.gov Identifier:	NCT00941382 History of Changes
Other Study ID Numbers:	OB Sil-02
Study First Received:	July 14, 2009
Last Updated:	July 15, 2009
Health Authority:	Mexico: Federal Commission for Sanitary Risks Protection

Keywords provided by Laboratorios Silanes S.A. de C.V.:

- sibutramine
- metformin
- obesity

Additional relevant MeSH terms:

- Obesity
- Overnutrition
- Nutrition Disorders
- Overweight
- Body Weight
- Signs and Symptoms
- Metformin
- Sibutramine
- Hypoglycemic Agents
- Physiological Effects of Drugs
- Pharmacologic Actions
- Appetite Depressants
- Anti-Obesity Agents
- Central Nervous System Agents
- Therapeutic Uses
- Antidepressive Agents
- Psychotropic Drugs

ClinicalTrials.gov processed this record on September 09, 2010


NATIONAL INSTITUTES OF HEALTH
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Study Of Comparative Effects Of Oral Clonidine Vs Oral Diazepam Pre-Medication On The Extent And Duration Of Sensory Blockade In Patients Undergoing Vaginal Hysterectomy Under Spinal Anaesthesia.

Internet Journal of Anesthesiology, 2009 by Namrata Toshniwal, Alka Halbe, Hemlatha Iyyer

Clonidine stimulates alpha 2 adrenergic inhibitory neurons in medullary vasomotor centre which decreases sympathetic outflow. Decreased sympathetic nervous system activity is manifested as decreases in systemic blood pressure, heart rate and cardiac output. Our results show that there was significant difference in the time of onset of anaesthesia, which coincides with the study done by Herbhej singh, George, Y .Gaines and Paul .F.White, in which they concluded that oral Clonidine shortened the onset time of tetracaine's sensory block & prolonged the duration of sensory & motor block. There are more studies, which also show that oral clonidine premedication prolong the sensory as well as motor blockade from Lignocaine & Tetracaine spinal anaesthesia. The antinociceptive effect produced by the orally administered 2- adrenergic agonist is mainly caused by direct spinal activation due to spread of the drug via the systemic circulation into the spinal cord. Neuraxial Clonidine inhibits spinal substance P release and nociceptive neuron firing produced by noxious stimuli. Clonidine modifies function of K channels in the CNS causing cell membrane hyperpolarization which decreases anaesthetic requirements.

Keywords: Spinal anesthesia; Clonidine; Analgesia

Introduction

Clonidine stimulates alpha 2 adrenergic inhibitory neurons in medullary vasomotor centre which decreases sympathetic outflow. Decreased sympathetic nervous system activity is manifested as decreases in systemic blood pressure, heart rate and cardiac output. Our results show that there was significant difference in the time of onset of anaesthesia, which coincides with the study done by Herbhej singh, George, Y .Gaines and Paul .F.White, in which they concluded that oral Clonidine shortened the onset time of tetracaine's sensory block & prolonged the duration of sensory & motor block. There are more studies, which also show that oral clonidine premedication prolong the sensory as well as motor blockade from Lignocaine & Tetracaine spinal anaesthesia. The antinociceptive effect produced by the orally administered 2- adrenergic agonist is mainly caused by direct spinal activation due to spread of the drug via the systemic circulation into the spinal cord. Neuraxial Clonidine inhibits spinal substance P release and nociceptive neuron firing produced by noxious stimuli. Clonidine modifies function of K channels in the CNS causing cell membrane hyperpolarization which decreases anaesthetic requirements.

METHODS

After obtaining approval from institutional ethics committee and written informed consent from all patients, this prospective and randomized study was carried out in 60 ASA grade I and II patients scheduled for vaginal hysterectomy in Dept of Anesthesiology, TNMC and Nair Hospital, Mumbai

All Patients were assessed on the previous day of the surgery and patient satisfying the inclusion criteria were included in the study.

Procedure, its complications and alternative methods were explained to the patient in his own language and patients consent was taken.

Criteria for inclusion:

1. Age : 18-60 yrs

2. Weight : 40-70kg

3. ASA : Grade I & II

4. Concious Co — operative patient

Criteria for exclusion:

1. Consent not available

2. Age <18 or >60 yrs

3. Weight < 40 or >70 kg

4. ASA grade III, IV, & V

5. Any contra — indication to spinal anaesthesia (Absolute or relative)

6. Non — co operative patient

7. Patients who are on antihypertensive or any sedative or on any antipsychotic drugs.

Base line record of pulse rate (by cardio scope), Blood pressure (by sphygmomanometer and NIBP)), Spo[sub 2] (by pulse oximetry) and respiratory rate were taken as Tbase.

In our study groups age and also physical parameters like weight and height were comparable among the two groups. There was no significant difference in preoperative parameters like pulse rate, respiratory rate and mean arterial pressure between the two groups.

The patients were randomly divided in two groups- Group C & Group D of 30 each. Patient in Group C received Clonidine 4 -5mcg/kg oral premedication and patients in Group D received Diazepam 0.20-0.25mg/kg oral premedication 90 minutes before spinal anaesthesia.

Blinding was done by packing the three tablets of 100mcg each of clonidine and three tablets of 5mg each of Diazepam in silver foil, subsequently the packets were placed in small plastic pouches and were numbered randomly as per computer generated number. Person dispensed the drug and person observed did not know the content of the packet. Decoding of packets was done at the end of the study.

After preloading, under all aseptic precautions with patient in sitting position, spinal anaesthesia was given with 23 G Quincke needle in L3-4 interspace with 2.5 cc of 0.5 % Bupivacaine and 25mcg Fentanyl. Patient was made to sit for 2 minutes after subarachnoid block and then made supine. Onset, duration, height of sensory block, time taken to reach highest level, and the time taken for two segment regression, time taken for four segment regression and the time when patient asks for analgesia were monitored and noted sensory blocked were evaluated by pinprick sensation.

Onset of anaesthesia was considered as appearance of analgesia at L1.

Duration of analgesia was considered as the time between onset and the time when patient asked for analgesia.

After operation patient were observed till sensory level weaned upto L1 and patient remained in the Gynaec recovery till patient received first dose of analgesia and that time was noted.

Results

The mean age in Group C was 50.93 years with standard deviation of 5.343 years and that in Group D was 50.93 years with standard deviation of 4.877 years. The groups were comparable according to age, weight and height.

Mean arterial pressure (MAP) was significantly lower in Group C as compared to Group D. Similar trends in falling Mean and Diastolic blood pressure are seen as with systolic blood pressure and the results were significant with lower blood pressure with Clonidine as compared to Diazepam.

According to above Table no 3, there was significant difference in time for onset of anaesthesia for Groups C and D. The mean time for onset of anaesthesia for Group C was 6.73 min with standard deviation of 2.392min and that for Group D was 8.50 min with standard deviation of 2.432 min. (p value 0.006). Our results demonstrate that there was significant difference in time for onset of anaesthesia for Groups C & D.

There was also a significant difference in time taken to reach highest sensory level in Group C and D. The mean time taken to reach highest level for Group C was 18.97min with standard deviation of 6.239min and that for Group D was 24.40 min with standard deviation of 6.026min. (p value 0.001).

The mean time taken for two segment regressions in Group C was 103.87 min with standard deviation of 12.754 min and that with Group D was 90.53 mins with standard deviation of 17.419 min. (p value 0.001)

The mean time taken for four segment regressions in Group C was 140.67 min with standard deviation of 27.753 min and that with Group D was 122.83 min with standard deviation of 24.589 min. (p value 0.001)

The mean time when patient asks for analgesia in Group C was 286.67 with standard deviation of 79.017min and that with Group D was 114.30 min with standard deviation of 15.234 min.The difference was significant. (p value 0.001)

The time for surgery with Group C was95.00 with standard deviation of 6.823 min and that for Group D was 96.17 min with standard deviation of 7.391. The difference was non-significicant. (p value 0.528)

Discussion

Clonidine is rapidly absorbed after oral administration. Peak plasma concentration is rapidly achieved in 60-90 mins is highly lipid soluble, easily crosses blood -brain barrier and therefore may interact with alpha -adrenergic receptors at spinal and supraspinal sites within the central nervous system.In addition previous studies suggest that clonidine may also affect peripheral sensory nerves as a sole agent or in combination with local anaesthetics.

Clonidine has been demonstrated to inhibit neurotransmission in both A-delta and C nerve fiber which are theorized to mediate pin-prick, surgical pain. Finally Clonidine has been demonstrated to potentiate inhibitory effects of local anaesthetics on C fiber activity. Therefore Clonidine may exert its effects within the central nervous system at peripheral nerve roots by potentiation of effects of local anaesthetics.

We have compared our results with previous study which also showed the same results.[1][2][3][4][5][6]. The primary mechanism of Clonidine analgesia is via a non -opoid spinal action on central alpha 2 adrenergic receptor in the dorsal horn of spinal cord.

The analgesic an effect of clonidine is mediated by the same central alpha2 adrenoreceptors that mediated its hypotensive effects. Clonidine added to local anaesthetics enhances the effects of local anaesthetics on C fiber action potentials.

We have also studies showing that prolongation of sensory anaesthesia when clonidine and fentanyl was combined was solely due to clonidine[1].

Our results showed that premedication with 4-5gm/ kg oral clonidine premedication prolongs the duration of sensory blockade by Bupivacaine and Fentanyl spinal anaesthesia as compared to that of 0.20-0.25mg/kg Diazepam oral premedication, and this results agree with the study done in 1992, by Kouechi Ota, Akiyoshi Namiki, Yoshihito Ujike & Ikuko Takahashi 3 . They concluded that prolongation of tetracaine sensory analgesia may be produced by premedication with oral clonidine premedication may have a distinct advantage because of its capacity to prolong sensory blockade & its potent sedating properties.

We added fentanyl to bupivacaine to determine its effect on anesthesia quality, and sensory block. The administration of intrathecal opioids may provide benefits in augmenting sensory level, but also carries a risk of respiratory depression but we had taken care of it by watching respiratory rate and saturation.

Our results showed that there was significant difference in the time of onset of anaesthesia, which coincides with the study done by Herbhej singh, George, Y .Gaines and Paul .F. White[1], in which they concluded that oral clonidine shortened the onset time of tetracaine's sensory block & prolonged the duration of sensory & motor block.

There are more studies, which also show that oral clonidine premedication prolong the sensory as well as motor blockade from Lignocaine & Tetracaine spinal anaesthesia. The antinociceptive effect produced by the orally administered 2-adrenergic agonist is mainly caused by direct spinal activation due to spread of the drug via the systemic circulation into the spinal cord. Neuraxial Clonidine inhibits spinal substance P release and nociceptive neuron firing produced by noxious stimuli. Clonidine modifies function of K channels in the CNS causing cell membrane hyperpolarization which decreases anaesthetic requirements.

The dose of clonidine (4-5gm/ kg) & time Interval (90min before spinal anaesthesia) were decided according to previous studies regarding safety of clonidine premedication in elderly & dose response studies of oral clonidine for tetracaine spinal anaesthesia.

Thus in the end as per results from our comparative study of effect of oral clonidine versus oral diazepam premedication on sensory blockade by intrathecal bupivacaine 0.5%(2.5ml) and fentanyl 25mcg, showed that clonidine hastens the onset of action, and reduces the time taken to reach highest sensory level.Clonidine also prolongs the total duration of sensory block by increasing the time for 2 and 4 segment sensory regression, also there was significant extension of analgesia.

Although few incidences of hypotension, bradycardia, nausea, vomiting and pruritus (Diazepam) were noted with both the groups, the difference was not statistically significant.

Endnotes

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PHOTO (BLACK & WHITE): Table 1: Comparison of Mean Age, W eight and Height

PHOTO (BLACK & WHITE): Table 2: Comparison of various pre-operative variables

PHOTO (BLACK & WHITE): Table 3: Comparison of various sensory block related parameters

Citation:

N. Toshniwal, A. Halbe & H. Iyyer : Study Of Comparative Effects Of Oral Clonidine Vs Oral Diazepam Pre-Medication On The Extent And Duration Of Sensory Blockade In Patients Undergoing Vaginal Hysterectomy Under Spinal Anaesthesia . The Internet Journal of Anesthesiology. 2009 Volume 19 Number 2

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Lexile Reading Level: 1620
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Pazopanib Plus Lapatinib Compared To Lapatinib Alone In Subjects With Inflammatory Breast Cancer

This study is currently recruiting participants.
Verified by GlaxoSmithKline, July 2010

First Received: November 9, 2007 Last Updated: July 8, 2010 History of Changes

Sponsor:	GlaxoSmithKline
Information provided by:	GlaxoSmithKline
ClinicalTrials.gov Identifier:	NCT00558103

Purpose

The double blind part of the study is being conducted to compare the efficacy and safety of pazopanib in combination with lapatinib with that of lapatinib alone in subjects with inflammatory breast cancer whose tumors overexpress the ErbB2 protein. There is also an Open-label pazopanib arm to this study designed to test whether pazopanib given alone and lapatinib given alone would be safe and effective to treat patients with inflammatory breast cancer.

Condition	Intervention	Phase
Inflammatory Breast Cancer	Drug: lapatinib (Tykerb) Drug: pazopanib (GW786034) Drug: Pazopanib	Phase III

Study Type: Interventional
Study Design: Allocation: Randomized
Endpoint Classification: Safety/Efficacy Study
Intervention Model: Parallel Assignment
Masking: Double Blind (Subject, Investigator)
Primary Purpose: Treatment

Official Title: A Randomized, Multicenter, Phase III Study Comparing the Combination of Pazopanib and Lapatinib Versus Lapatinib Monotherapy in Patients With ErbB2 Over-expressing Inflammatory Breast Cancer

Resource links provided by NLM:

Genetics Home Reference related topics: breast cancer

MedlinePlus related topics: Breast Cancer Cancer

Drug Information available for: Lapatinib Lapatinib Ditosylate Pazopanib

U.S. FDA Resources

Further study details as provided by GlaxoSmithKline:

Primary Outcome Measures:

- Progression-free survival at anytime. [Time Frame: on going]

Secondary Outcome Measures:

- Overall Response Rate (ORR)Overall survival (OS)Safety and tolerabilityHealth Status Assessments [Time Frame: on going]

Estimated Enrollment:	360
Study Start Date:	December 2007
Estimated Study Completion Date:	June 2012
Estimated Primary Completion Date:	June 2012 (Final data collection date for primary outcome measure)

Arms	Assigned Interventions
arm 1: Active Comparator	Drug: lapatinib (Tykerb) comparator Drug: pazopanib (GW786034) comparator
Pazopanib Open-label: Active Comparator Pazopanib alone arm incorporated into study VEG108838 (lapatinib + pazopanib vs. lapatinib monotherapy in patients with recurrent Her2+ IBC).	Drug: Pazopanib Pazopanib monotherapy

Eligibility

Ages Eligible for Study:	18 Years and older
Genders Eligible for Study:	Female
Accepts Healthy Volunteers:	No

Criteria

Inclusion criteria:

Specific information regarding warnings, precautions, contraindications, adverse events, and other pertinent information on the investigational product that may impact patient eligibility is provided in the pazopanib IB and lapatinib prescribing information (or the lapatinib IB).

For Cohort 1 of this study, eligible patients met inclusion criteria outlined in the original version of the protocol and protocol amendment 1.

For Cohort 2 of this study, eligible patients must meet all of the following criteria:

- Patients must have evaluable Inflammatory Breast Cancer (IBC) substantiated by all of the following prior to randomization:
- History of invasive breast cancer documented by a biopsy and accompanying pathology report
- Current photographs* (global view and close-up views of all skin lesions) submitted at screening demonstrating unequivocal evidence of IBC as determined by either the medical monitor alone or in consulation with one or more of the study Principal Investigators.
- All patients must have photography at screening. Canfield Scientific Inc. will provide centralized monitoring, tracking, and collection of patients' photographs throughout the study. Screening photographs must be uploaded to the Canfield Scientific Inc website and approved by Canfield Scientific Inc, as the central photography vendor. The photographs, along with the completed Inflammatory Breast Cancer Skin Assessment Tool (IBSAT), must be reviewed and approved by GSK before a patient can be randomized. Sites should allow a

minimum of 3 business days for this process. Sites submitting quality photographs and IBSATs on a regular basis will receive an exemption from this requirement for future patients.

- Patients with secondary IBC are eligible.
- Measurable lesions (cutaneous or radiographic) may be in the field of prior standard or palliative radiation therapy; however, there must be at least a 4 week period between the last radiation treatment and the baseline scan documenting disease status for the lesion to be measurable. If the irradiated lesion is the only site of disease, documented progression of the irradiated lesion is required.
- Disease progression or relapse following treatment for invasive breast cancer, which must have included a chemotherapy regimen. In regions where trastuzumab is available with no barriers to access*, patients must have received prior trastuzumab in addition to chemotherapy in order to be eligible. * (Barriers to access may include financial considerations.)
- Unequivocal ErbB2 overexpressing breast cancer, defined as 3+ staining by immunohistochemistry (IHC), or 2+ staining by IHC in conjunction with ErbB2 gene amplification by FISH/CISH, or ErbB2 gene amplification by FISH/CISH alone (in subjects whose tumor blocks were not assessed by IHC). ErbB2 gene amplification is defined by: > six (6) ErbB2 gene copies/nucleus for test systems without an internal control probe or an ErbB2/CEP 17 ratio of more than 2.2.

Sites must submit a copy of the laboratory report demonstrating unequivocal ErbB2 overexpression, if testing performed at a local laboratory, with the screening worksheet. Archived tumor must be provided for all patients for ErbB2 FISH testing by the central laboratory. Patients will remain on study based on local ErbB2 expression results. If archived tumor is not available, a biopsy must be obtained at screening and sent to TMD Laboratoraties for ErbB2 FISH testing.

- Patients must provide written informed consent prior to performance of study-specific procedures or assessments, and must be willing to comply with treatment and follow up. Procedures conducted as part of the patient's routine clinical management (e.g., blood count, imaging study) and obtained prior to signing of informed consent may be utilized for screening or baseline purposes provided these procedures are conducted as specified in the protocol.

Note: Informed consent may be obtained prior to the protocol-specified screening window (i.e. Day -14 to Day -1).

- Females age ≥ 18 years, except in Tunisia. In Tunisia, patients must be ≥ 20 years to be eligible for this study.
- Adequate organ function as defined below:
- System (Laboratory Values)
- Hematologic:Absolute neutrophil count (ANC)(≥ 1.5 X 10^9/L)Hemoglobin1(≥9 g/dL)Platelets (≥100 X 10^9/L)International normalized ratio (INR)(≤ 1.2 X upper limit of normal (ULN)) Partial thromboplastin time (PTT)(≤1.2 X ULN)
- Hepatic:Total bilirubin2 (≤ 1.5 X upper limit of normal (ULN))AST and ALT(≤ 2.5 X ULN)
- Renal:Serum Creatinine (≤ 1.5 mg/dL)Or, if serum creatinine >1.5 mg/dL,
- Calculated creatinine clearance(≥50 mL/min)
- Urine Protein to Creatinine Ratio(<1)
- Patients may not have had a transfusion within 7 days of screening assessment.
- Exception: Patients with elevated bilirubin levels due to Gilberts syndrome are eligible.
- Cardiac ejection fraction within the institutional range of normal as measured by echocardiogram. MUGA scans will be accepted in cases where an echocardiogram cannot be performed or is inconclusive or where MUGA scans are the accepted standard. Patients with known history of uncontrolled or symptomatic angina, arrhythmias, or congestive heart failure are not eligible.
- Eastern Cooperative Oncology Group (ECOG) performance status of 0-2.
- A female is eligible to enter and participate in this study if she is of:

Non-childbearing potential (i.e., physiologically incapable of becoming pregnant), including any female who has had:

- A hysterectomy
- A bilateral oophorectomy (ovariectomy)

- A bilateral tubal ligation
- Is post-menopausal
- Patients not using hormone replacement therapy (HRT) must have experienced total cessation of menses for ≥ 1 year and be greater than 45 years in age, OR, in questionable cases, have a follicle stimulating hormone (FSH) value >40 mIU/mL and an estradiol value < 40pg/mL (<140 pmol/L).

Patients must discontinue HRT prior to study enrollment due to the potential for inhibition of CYP enzymes that metabolize estrogens and progestins (See Section 8). For most forms of HRT, at least 2-4 weeks must elapse between the cessation of HRT and determination of menopausal status; length of this interval depends on the type and dosage of HRT. If a female patient is determined not to be post-menopausal, they must use adequate contraception, as defined immediately below.

Childbearing potential, including any female who has had a negative serum pregnancy test within 2 weeks prior to the first dose of study treatment, preferably as close to the first dose as possible, has used adequate contraception since the pregnancy test and agrees to use adequate contraception as described below. GSK acceptable contraceptive methods, when used consistently and in accordance with both the product label and the instructions of the physician, are as follow:

- An intrauterine device with a documented failure rate of less than 1% per year.
- Vasectomized partner who is sterile prior to the female patient's entry and is the sole sexual partner for that female.
- Complete abstinence from sexual intercourse for 14 days before exposure to investigational product, through the dosing period, and for at least 21 days after the last dose of investigational product.
- Double-barrier contraception (condom with spermicidal jelly, foam suppository, or film; diaphragm with spermicide; or male condom and diaphragm with spermicide).

Note: Oral contraceptives are not reliable due to potential drug drug interactions.

Female patients who are lactating should discontinue nursing prior to the first dose of investigational product and should refrain from nursing throughout the treatment period and for 14 days following the last dose of investigational product.

- French patients: In France, a patient will be eligible for inclusion in this study only if either affiliated to or a beneficiary of a social security category.

Exclusion Criteria:

- Patients meeting any of the following criteria must not be enrolled in the study:
- Treatment in the 14 days prior to randomization with any cancer therapy (tumor embolization, chemotherapy, immunotherapy, biological therapy, or hormonal therapy) or treatment with mitomycin within 6 weeks prior to randomization. Such treatment may not be resumed or begun after study entry. Note: Patients receiving LH-RH analogue therapy prior to the study may continue to receive LH-RH analogues for the duration of study participation. Bisphosphonates are permitted if started prior to Day 1.
- Any ongoing toxicity from prior anti-cancer therapy that is >Grade 1 and/or that is progressing in severity (with the exception of alopecia).
- Prior lapatinib therapy or other Her2/ErbB2 targeted therapy (except trastuzumab).
- Prior therapy with an anti-VEGF antibody or other VEGF/VEGF-R targeted therapy.
- Use of an investigational agent, including an investigational anti-cancer agent, within 28 days or 5 half-lives, whichever is longer, prior to the first dose of investigational product.
- Use of any prohibited medication within the timeframes listed in Section 8.1.3
- History of another malignancy.
- Note: Subjects who have had another malignancy and have been disease-free for 5 years, or subjects with a history of completely resected non-melanomatous skin carcinoma or successfully treated in situ carcinoma are eligible. If subject previously had breast cancer, it must have been HER2+ with either 3+ overexpression by IHC or unequivocal HER2 gene amplification by FISH or CISH.
- History or clinical evidence of central nervous system (CNS) metastases or leptomeningeal carcinomatosis, except for individuals who have previously-treated CNS metastases, are asymptomatic, and have had no requirement for steroids or anti-seizure medication for 2

months prior to first dose of study drug. Screening with CNS imaging studies (computed tomography [CT] or magnetic resonance imaging [MRI]) is required only if clinically indicated or if the subject has a history of CNS metastases.

- Clinically significant gastrointestinal abnormalities that may increase the risk for GI bleeding including, but not limited to:
- Active peptic ulcer disease
- Known intraluminal metastatic lesion/s with suspected bleeding
- Inflammatory bowel disease
- Ulcerative colitis, or other gastrointestinal conditions with increased risk of perforation
- History of abdominal fistula, gastrointestinal perforation, or intra abdominal abscess within 28 days prior to beginning study treatment.
- Clinically significant gastrointestinal abnormalities that may affect absorption of investigational product including, but limited to:
- Malabsorption syndrome
- Major resection of the stomach or small bowel.
- Presence of uncontrolled infection.
- Prolongation of corrected QT interval (QTc) > 480 msecs.
- History of any one or more of the following cardiovascular conditions within the past 6 months:
- Cardiac angioplasty or stenting
- Myocardial infarction
- Unstable angina
- Arterial thrombosis
- Symptomatic peripheral vascular disease
- Class III or IV congestive heart failure, as defined by the New York Heart Association (NYHA) (see Section 15.9 Appendix 9 for description).
- Poorly controlled hypertension [defined as systolic blood pressure (SBP) of ≥140mmHg or diastolic blood pressure (DBP) of ≥ 90mmHg].

Note: Initiation or adjustment of antihypertensive medication(s) is permitted during the screening period, in order to control a patient's BP prior to randomization. Blood pressure must be re-assessed on two occasions that are separated by a minimum of 1 hour. The mean SBP / DBP values from each blood pressure assessment must be < 140/90mmHg in order for a patient to be eligible for the study. See Section 6.2 and Section 6.4.2 for details on blood pressure control and reassessment prior to study enrollment.

- History of cerebrovascular accident, including TIA, pulmonary embolism or deep venous thrombosis (DVT).
- Prior major surgery or trauma within 28 days prior to first dose of investigational product and/or presence of any non-healing wound, fracture, or ulcer (other than ulcers due to inflammatory breast cancer).
- Evidence of active bleeding or bleeding diathesis.
- Hemoptysis within 6 weeks prior to first dose of investigational product.
- Known endobronchial lesions or involvement of large pulmonary vessels by tumor.
- Any serious and/or unstable pre-existing medical, psychiatric, or other condition that could interfere with patient's safety, provision of informed consent, or compliance to study procedures.
- Known immediate or delayed hypersensitivity reaction or idiosyncrasy to drugs chemically related to pazopanib or lapatinib.
- Have current active hepatic or biliary disease (with exception of patients with Gilbert's syndrome, asymptomatic gallstones, liver metastases or stable chronic liver disease per investigator assessment).

Contacts and Locations

Please refer to this study by its ClinicalTrials.gov identifier: NCT00558103

Contacts

Contact: US GSK Clinical Trials Call Center 877-379-3718

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Sponsors and Collaborators

GlaxoSmithKline

Investigators

Study Director: GSK Clinical Trials GlaxoSmithKline

More Information

No publications provided

Responsible Party:	GSK (Study Director)
ClinicalTrials.gov Identifier:	NCT00558103 History of Changes
Other Study ID Numbers:	VEG108838
Study First Received:	November 9, 2007
Last Updated:	July 8, 2010
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- Her2
- ErbB2
- GW786034
- GW572016
- Skin
- Cutaneous Disease
- RECIST
- Inflammatory
- Breast Cancer
- Pazopanib
- Lapatinib

Additional relevant MeSH terms:

- Breast Neoplasms
- Neoplasms by Site
- Neoplasms
- Breast Diseases
- Skin Diseases
- Lapatinib
- Protein Kinase Inhibitors
- Enzyme Inhibitors
- Molecular Mechanisms of Pharmacological Action
- Pharmacologic Actions
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Dronedarone is less effective, but safer than amiodarone in atrial fibrillation

On the Horizon Rapid Review

NHS NPC National Prescribing Centre

27 October 2009,

An indirect comparison meta-analysis found that dronedarone was significantly less effective than amiodarone in preventing recurrence of AF, but was associated with fewer side effects requiring discontinuation. It was predicted that for every 100 people treated for one year for AF with dronedarone rather than amiodarone, there would be 23 more recurrences of AF and 6 fewer adverse events requiring discontinuation.

Level of evidence
Level 2 (limited quality, patient-orientated evidence) according to the SORT criteria

Action
Dronedarone may be launched in the UK by the end of 2009. It is likely to be indicated in clinically stable adult patients with history of, or current non-permanent atrial fibrillation (AF) to prevent recurrence of AF or to lower ventricular rate. Contraindications, special warnings, and monitoring requirements (as will be described in the summary of product characteristics [SPC]) are not yet published.

The exact place in therapy of dronedarone is uncertain. Until NICE guidance on dronedarone is published (due June 2010) prescribers should continue to follow the existing NICE guideline for the management of AF. Dronedarone could be considered as an option, **within its licensed indications, by specialists** for those patients for whom amiodarone is indicated, but not appropriate, e.g. if there are intolerable adverse effects. Clinicians will need to balance whether the use of dronedarone — a less efficacious but possibly safer antiarrhythmic drug than amiodarone — is justified for their patients with AF. For patients who are already receiving and tolerating amiodarone and have not developed unacceptable side effects, there would appear to be no good reason to switch to dronedarone.

Dronedarone is likely to be significantly more expensive than currently used anti-arrhythmic drugs, which are mainly available generically. Local decision making bodies on medicines are advised to

engage with stakeholders and agree a protocol for use when dronedarone is launched. This includes identifying those patients for whom the drug may be appropriate and planning for possible NICE guidance. It is important that steps are taken to inform prescribers of any contraindications and precautions, to ensure that dronedarone is used appropriately.

What is the background to this?
On 24 September 2009, the EMEA Committee for Medicinal Products for Human Use (CHMP) adopted a positive opinion for dronedarone (Multaq®). It recommended granting a marketing authorisation for adult clinically stable patients with history of, or current non-permanent AF, to prevent recurrence of AF or to lower ventricular rate. A key study considered in providing this opinion was the ATHENA trial, which we have reviewed in a previous blog. In the ATHENA trial of 4,628 patients with AF or flutter, dronedarone was more effective than placebo (mean follow-up of 21 months) in reducing cardiovascular (CV) hospital admissions or death from any cause (31.9% vs. 39.4%; hazard ratio 0.76, 95% confidence interval (CI) 0.69 to 0.84, P<0.001), as well as other trials.

Because of the limited information available from studies that directly compare the efficacy and safety of dronedarone with amiodarone, Piccini and colleagues carried out a systematic review and indirect comparison meta-analysis using data from placebo-controlled trials of the two drugs in over 6000 people with AF.

Further information about AF can be found on the cardiovascular floor of NPCi. Dronedarone has been reviewed in an On the Horizon bulletin. Dronedarone is part of the 19th wave of technology appraisals from NICE, but a NICE clinical guideline on the management of AF is already available.

What does this study claim?
The study claims that dronedarone is less effective than amiodarone for the maintenance of sinus rhythm, but has fewer side effects.

The meta-analysis identified a statistically significant estimated reduction in recurrent AF with amiodarone versus placebo (odds ratio [OR] 0.12; 95%CI 0.08 to 0.19) but not for dronedarone versus placebo (OR 0.79; 95% CI 0.33 to 1.87). Using a logistic regression model incorporating all trial evidence, amiodarone was found to be superior to dronedarone (OR 0.49; 95% CI 0.37 to 0.63; P<0.001) for the prevention of recurrent AF, but was more likely to result in adverse events requiring drug discontinuation (OR 1.81; 95% CI 1.33 to 2.46; P<0.001).

The authors also suggest a 'trend' for reduced mortality in favour of dronedarone, although no statistically significant difference between treatments was identified (P=0.066).

How does this relate to other studies?
These results are consistent with the results of the DIONYSOS trial that directly compared dronedarone with amiodarone for the maintenance of sinus rhythm. However, results of DIONYSOS are only reported in a press release and are yet to be published in a peer-reviewed journal. DIONYSOS was a study of 504 patients with AF (mean follow-up 7 months). Dronedarone was less effective than amiodarone in preventing AF recurrence, or withdrawal due to intolerance or lack of efficacy (74% vs. 55%, P<0.001). Fewer thyroid and neurological events were reported in the dronedarone patients, but there were more reports of diarrhoea, vomiting and nausea. Amiodarone caused more bradycardia and QT prolongation. No cases of Torsade de Pointes were reported.

So what?
The present study found that dronedarone was significantly less effective in preventing recurrence of AF, but was associated with fewer side effects leading to discontinuation than with amiodarone. The indirect meta-analytical approach used in this study has many limitations (see accompanying

Editorial) and the findings of this study can only be considered hypothesis generating and require confirmation from direct comparisons in adequately powered trials. Nevertheless, the reduction in efficacy and reduction in adverse effects seen in this study are consistent with the preliminary results from DIONYSOS.

Although the ATHENA study identified a significant benefit for preventing hospitalisation for CV events or death for dronedarone over placebo, no benefit over amiodarone has yet been demonstrated in this regard. Whether or not dronedarone offers any particular advantage over amiodarone for an individual with AF will require a value judgement of whether likely benefits from reduced side effects outweigh the disadvantage of a shorter time to recurrence of AF. At present there is no good quality clinical evidence from comparative studies measuring important patient-oriented outcomes (e.g. quality of life) demonstrating whether or not dronedarone offers any net clinical benefit over amiodarone at a population level.

NICE guidelines on the management of atrial fibrillation detail those situations where amiodarone might be considered. However, there are many other approaches that should be considered before, or as alternatives to, the use of amiodarone.

Full prescribing details (including dosage, contraindications, drug and food interactions, and monitoring requirements) for dronedarone have yet to be published. As discussed in a previous blog of the ATHENA trial, it may be advisable not to initiate therapy with dronedarone in patients with severe heart failure and left ventricular dysfunction.

Dronedarone is already licensed for use in the US. Contraindications in the US include patients with severe heart failure or those with NYHA 2 or 3 heart failure with a recent decompensation requiring hospitalisation or referral to a specialised heart failure clinic. Increases in serum creatinine have occurred in clinical trials with dronedarone, and there are potentially important drug interactions to consider (e.g. with strong CYP3A inhibitors such as voriconazole, and with drugs that prolong the QT interval, which might increase the risk of Torsade de Pointes). It is important that steps are taken to inform prescribers of any contraindications and precautions, to ensure that dronedarone is used and monitored appropriately.

Study details

Piccini JP, Hasselblad V, Peterson ED, et al. Comparative efficacy of dronedarone and amiodarone for the maintenance of sinus rhythm in patients with atrial fibrillation. J Am Coll Cardiol 2009;54:1089–95

Design: Systematic review and indirect comparison meta-analysis of RCTs of amiodarone (4 studies) and dronedarone (4 studies) versus placebo for the prevention of AF.

Patients: The dronedarone and amiodarone trials included 5,967 and 669 patients respectively. The mean age across all trials was 65 years. Studies of subjects age <18 years and subjects with acute cardioversion, catheter ablation, and post-operative AF were excluded. In all 4 dronedarone trials, patients with permanent AF were excluded. Additional exclusion criteria included advanced symptomatic heart failure, a corrected QT interval >500ms, and bradycardia with a heart rate <50 beats/min. In contrast to the dronedarone trials, the amiodarone trials predominantly included patients with persistent and permanent AF.

Interventions: Dronedarone or amiodarone versus placebo. All trials had a follow up of at least 6 months (means of 13 and 16 months, respectively).

Comparison: The effect of amiodarone versus dronedarone was summarised by the use of indirect comparison meta-analysis and normal logistic meta-regression models.

Outcomes and Results:
There was a significant estimated reduction in recurrent AF with amiodarone versus placebo (OR 0.12; 95%CI 0.08 to 0.19) but not dronedarone versus placebo (OR 0.79; 95%CI 0.33 to 1.87). A normal logistic regression model incorporating all trial evidence found amiodarone superior to dronedarone (OR 0.49; 95%CI 0.37 to 0.63; P<0.001) for the prevention of recurrent AF. There was no statistically significant difference between amiodarone and dronedarone identified with regard to all-cause mortality (OR 1.61; 95% CI 0.97 to 2.68; P=0.066). More patients discontinued treatment because of adverse effects with amiodarone than with dronedarone (OR 1.81; 95%CI 1.33 to 2.46; P<0.001). For every 1,000 patients treated with dronedarone instead of amiodarone, it was estimated that there would be approximately 228 more recurrences of AF in exchange for 62 fewer adverse events requiring discontinuation of drug.

Sponsorship: The lead author is supported by an American College of Cardiology Foundation/Merck award.

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'Trusted sources' of evidence

- BNF
- Clinical Evidence (BMJ)
- Clinical Knowledge Summaries (aka Prodigy)
- Cochrane
- Drug and Therapeutics Bulletin
- Electronic Medicines Compendium (EMC)
- Evidence Based Medicine Journal
- MeReC
- MHRA Drug Safety Update
- NICE – National Institute for Health and Clinical Excellence
- Scottish Medicines Consortium (SMC)
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Other blogs of interest

- Bad Science
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- Matt Robinson's Prescribing Adviser Blog

Other Health Links

- Department of Health
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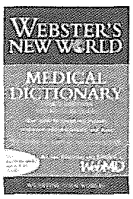

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Definition of Contraindication

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Contraindication: A condition which makes a particular treatment or procedure inadvisable. A contraindication may be absolute or relative.

- An absolute contraindication is a situation which makes a particular treatment or procedure absolutely inadvisable. In a baby, for example, aspirin is absolutely contraindicated because of the danger that aspirin will cause Reye syndrome.
- A relative contraindication is a condition which makes a particular treatment or procedure somewhat inadvisable but does not rule it out. For example, X-rays in pregnancy are relatively contraindicated (because of concern for the developing fetus) unless the X-rays are absolutely necessary.

A contraindication is literally contra- (against) an indication, against something that is indicated as advisable or necessary.

Last Editorial Review: 5/12/2004

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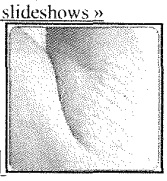

Scales, Plaques & Eruptions

233 HEART FAILURE

Eugene Braunwald

Heart failure is the pathophysiologic state in which an abnormality of *cardiac* function is responsible for the failure of the heart to pump blood at a rate commensurate with the requirements of the metabolizing tissues *and/or* can do so only from an abnormally elevated diastolic volume. Heart failure is frequently, but not always, caused by a defect in myocardial contraction, and then the term *myocardial failure* is appropriate. The latter may result from a primary abnormality in heart muscle, as occurs in the cardiomyopathies and in viral myocarditis (Chap. 239). Myocardial failure also may result from extramyocardial abnormalities, such as coronary atherosclerosis which leads to myocardial ischemia and infarction, as well as from abnormalities of the heart valves in which the heart muscle is damaged by the long-standing excessive hemodynamic burden imposed by the valvular abnormality, and/or by the rheumatic process (Chap. 236).

In other patients with heart failure, however, a similar clinical syndrome is present but without any detectable abnormality of *myocardial* function. In some of these patients the normal heart is suddenly presented with a mechanical load that exceeds its capacity, such as an acute hypertensive crisis, rupture of an aortic valve cusp, or massive pulmonary embolism. Heart failure, in the presence of normal myocardial function, also occurs in chronic conditions in which there is impairment of filling of the ventricles due to a mechanical abnormality such as tricuspid and/or mitral stenosis, constrictive pericarditis without myocardial involvement, endocardial fibrosis, and some forms of hypertrophic cardiomyopathy. In many patients with heart failure, particularly those with valvular or congenital heart disease, a combination of impaired myocardial function and mechanical abnormality exists.

Heart failure should be distinguished from (1) conditions in which there is circulatory congestion consequent to abnormal salt and water retention but in which there is no disturbance of cardiac function per se (the latter syndrome, termed the *congested state*, may result from the abnormal salt and water retention of renal failure or from excess parenteral administration of fluids and electrolytes) and (2) noncardiac causes of inadequate cardiac output, including shock due to hypovolemia and redistribution of blood volume (Chap. 38).

The ventricles respond to a chronically increased hemodynamic burden with the development of hypertrophy. With volume overload when the ventricle is called on to deliver an elevated cardiac output for prolonged periods, as in valvular regurgitation, it develops *eccentric hypertrophy*, i.e., cavity dilatation, with an increase in muscle mass so that the ratio between wall thickness and ventricular cavity size remains relatively constant. With chronic pressure overload, as in valvular aortic stenosis or untreated hypertension, it develops *concentric hypertrophy*, in which the ratio between wall thickness and ventricular cavity size increases. In both conditions, a stable hyperfunctioning state may exist for many years, but myocardial function may ultimately deteriorate, leading to heart failure. Heart failure represents a major public health problem in industrialized nations. It appears to be the only common cardiovascular condition that is increasing in prevalence and incidence. In the United States, heart failure is responsible for almost 1 million hospital admissions and 40,000 deaths annually. Since heart failure is more common in the elderly, its prevalence is likely to continue to increase as the population ages.

CAUSES OF HEART FAILURE

In evaluating patients with heart failure, it is important to identify not only the *underlying cause* of the heart disease but also the *precipitating cause* of heart failure. The cardiac abnormality produced by a congenital or acquired lesion such as valvular aortic stenosis may exist for many years and produce no clinical disability. Frequently, however, clinical manifestations of heart failure appear for the first time in the course of some acute disturbance that places an additional load on a myocardium that chronically is excessively burdened. The heart may be compensated but have little additional reserve, and the additional load imposed by a precipitating cause results in further deterioration of cardiac function. Identification of such precipitating causes is of critical importance because their prompt alleviation may be lifesaving. In the absence of underlying heart disease, these acute disturbances do not usually, by themselves, lead to heart failure.

PRECIPITATING CAUSES

1. *Infection.* Patients with pulmonary vascular congestion are also more susceptible to pulmonary infections; any infection may precipitate heart failure. The resulting fever, tachycardia, and hypoxemia and the increased metabolic demands may place a further burden on the overloaded, but compensated myocardium of a patient with chronic heart disease.

2. *Anemia.* In the presence of anemia, the oxygen needs of the metabolizing tissues can be met only by an increase in the cardiac output (Chap. 59). Although such an increase in cardiac output can be sustained by a normal heart, a diseased, overloaded, but otherwise compensated heart may be unable to augment sufficiently the volume of blood that it delivers to the periphery. In this manner, the combination of anemia and previously compensated heart disease can lead to inadequate oxygen delivery to the periphery and precipitate heart failure.

3. *Thyrotoxicosis and pregnancy.* As in anemia and fever, in thyrotoxicosis and pregnancy, adequate tissue perfusion requires an increased cardiac output. The development or intensification of heart failure may actually be one of the first clinical manifestations of hyperthyroidism in a patient with underlying heart disease that was previously compensated (Chap. 331). Similarly, heart failure not infrequently occurs for the first time during pregnancy in women with rheumatic valvular disease, in whom cardiac compensation may return following delivery.

4. *Arrhythmias.* In patients with compensated heart disease, arrhythmias are among the most frequent precipitating causes of heart failure. They exert a deleterious effect for a variety of reasons: (a) Tachyarrhythmias reduce the time period available for ventricular filling. In patients with ischemic heart disease, tachyarrhythmias also may cause ischemic myocardial dysfunction. (b) The dissociation between atrial and ventricular contractions characteristic of many arrhythmias results in the loss of the atrial booster pump mechanism, thereby raising atrial pressures. (c) In any arrhythmia associated with abnormal intraventricular conduction, myocardial performance may become further impaired because of the loss of normal synchronicity of ventricular contraction. (d) Marked bradycardia associated with complete atrioventricular block or other severe bradyarrhythmias reduces cardiac output unless stroke volume rises reciprocally; this compensatory response cannot occur with serious myocardial dysfunction even in the absence of heart failure.

5. *Rheumatic and other forms of myocarditis.* Acute rheumatic fever and a variety of other inflammatory or infectious processes affecting the myocardium may impair myocardial function in patients with or without preexisting heart disease (Chaps. 236 and 239).

6. *Infective endocarditis.* The additional valvular damage, anemia, fever, and myocarditis that often occur as a consequence of infective endocarditis may, singly or in concert, precipitate heart failure (Chap. 126).

7. *Physical, dietary, fluid, environmental, and emotional excesses.* The augmentation of sodium intake, the inappropriate discontinuation of medications to treat heart failure, blood transfusions, physical overexertion, excessive environmental heat or humidity, and emotional crises all may precipitate heart failure in patients with heart disease who were previously compensated.

8. *Systemic hypertension.* Rapid elevation of arterial pressure, as may occur in some instances of hypertension of renal origin or upon discontinuation of antihypertensive medication, may result in cardiac decompensation (Chap. 246).

9. *Myocardial infarction.* In patients with chronic but compensated ischemic heart disease, a fresh infarct, sometimes otherwise silent clinically, may further impair ventricular function and precipitate heart failure (Chap. 243).

10. *Pulmonary embolism.* Physically inactive patients with low cardiac output are at increased risk of developing thrombi in the veins of the lower extremities or the pelvis. Pulmonary emboli may result in further elevation of pulmonary arterial pressure, which in turn may produce or intensify ventricular failure. In the presence of pulmonary vascular congestion, such emboli also may cause pulmonary infarction (Chap. 261).

A systematic search for these precipitating causes should be made in every patient with the new development or recent intensification of heart failure, especially if it is refractory to the usual methods of therapy. If properly recognized, the precipitating cause of heart failure usually can be treated more effectively than the underlying cause. Therefore, the prognosis in patients with heart failure in whom a precipitating cause can be identified, treated, and eliminated is more favorable than it is in patients in whom the underlying disease process has advanced to the point of producing heart failure.

FORMS OF HEART FAILURE

Heart failure may be described as *systolic* or *diastolic*, *high-output* or *low-output*, *acute* or *chronic*, *right-sided* or *left-sided*, and *forward* or *backward*. These descriptors are often useful in a clinical setting, particularly early in the patient's course, but late in the course of chronic heart failure the differences between them often become blurred.

SYSTOLIC VERSUS DIASTOLIC FAILURE The distinction between these two forms of heart failure, described on p. 1284 and in Fig. 232-8, relates to whether the principal abnormality is the inability to contract normally and expel sufficient blood (systolic failure) or to relax and fill normally (diastolic failure). The major clinical manifestations of systolic failure relate to an inadequate cardiac output with weakness, fatigue, reduced exercise tolerance, and other symptoms of hypoperfusion, while in diastolic failure they relate principally to an elevation of filling pressures. In many patients, particularly those who have both ventricular hypertrophy *and* dilatation, abnormalities both of contraction and relaxation coexist.

Diastolic heart failure may be caused by increased resistance to ventricular inflow and reduced ventricular diastolic capacity (constrictive pericarditis and restrictive, hypertensive, and hypertrophic cardiomyopathy), impaired ventricular relaxation (acute myocardial ischemia, hypertrophic cardiomyopathy), and myocardial fibrosis and infiltration (dilated, chronic ischemic, and restrictive cardiomyopathy).

HIGH-OUTPUT VERSUS LOW-OUTPUT HEART FAILURE It is useful to classify patients with heart failure into those with a low cardiac output, i.e., *low-output heart failure*, and those with an elevated cardiac output, i.e., *high-output heart failure*. The former occurs secondary to ischemic heart disease, hypertension, dilated cardiomyopathy, and valvular and pericardial disease, while the latter is seen in patients with heart failure and hyperthyroidism, anemia, pregnancy, arteriovenous fistulas, beriberi, and Paget's disease. In clinical practice, however, low-output and high-output heart failure cannot always be readily distinguished. The normal range of cardiac output is wide [2.2 to 3.5 (L/min)/m^2], and in many patients with so-called low-output heart failure the cardiac output may actually be just within the normal range at rest (although it is lower than it had been previously), but it fails to rise normally during exertion. On the other hand, in patients with so-called high-output heart failure the output may not exceed the upper limits of normal (although it would have been elevated had it been measured before heart failure supervened), but rather it may have fallen to the upper limit of normal. Regardless of the *absolute* level of the cardiac output, however, cardiac failure may be said to be present when the characteristic clinical manifestations described below are accompanied by a depression of the curve relating ventricular end-diastolic volume to cardiac performance (see Fig. 232-6).

An integral physiologic component of *systolic* heart failure (p. 1284) is the delivery of an inadequate quantity of oxygen required by the metabolizing tissues. In the absence of peripheral shunting of blood, this is reflected in an abnormal widening of the normal arterial–mixed venous oxygen difference (35 to 50 mL/L in the basal state). In mild cases, such an abnormality may not be present at rest but becomes evident only during exertion or other hypermetabolic states. In patients with high cardiac output states, such as those associated with arteriovenous fistula or thyrotoxicosis, the arterial–mixed venous oxygen difference is normal or low. The mixed venous oxygen saturation is raised by the admixture of blood that has been diverted from the metabolizing tissues, and it may be presumed that even in these patients the delivery of oxygen to the latter is reduced despite the normal or even elevated mixed venous oxygen saturation. When heart failure occurs in such patients, the arterial–mixed venous oxygen difference, regardless of the absolute value, still exceeds the level that existed prior to the development of heart failure. Therefore, the cardiac output, though normal or even elevated, is lower than before heart failure supervened.

The mechanisms responsible for the development of heart failure in patients whose cardiac outputs are initially high are complex and depend on the underlying disease process. In most of these conditions the heart is called on to pump abnormally large quantities of blood in order to deliver the normal quota of oxygen to the metabolizing tissues. The burden placed on the myocardium by the increased flow load resembles that produced by chronic regurgitant valvular lesions. In addition, thyrotoxicosis and beriberi also may impair myocardial metabolism directly, while severe anemia may interfere with myocardial function by producing myocardial anoxia, especially in the presence of underlying obstructive artery disease.

ACUTE VERSUS CHRONIC HEART FAILURE The prototype of acute heart failure is the patient who is entirely well but who suddenly develops a large myocardial infarction or rupture of a cardiac valve. Chronic heart failure is typically observed in patients with dilated cardiomyopathy or multivalvular heart disease that develops or progresses slowly. Acute heart failure is usually largely systolic, and the sudden reduction in cardiac output often results in systemic hypotension without peripheral edema. In chronic heart failure, arterial pressure tends to be well maintained until very late in the course, but there is often accumulation of edema. Despite these obvious differences in clinical presentation, there is no fundamental distinction between acute and chronic heart failure. For example, intensive efforts to prevent expansion of blood volume by means of dietary sodium restriction and the administration of diuretics will frequently delay the development of exertional dyspnea and edema in patients with chronic valvular heart disease (i.e., it will mask the clinical manifestations of chronic heart failure) until an acute episode, such as an arrhythmia or infection, precipitates acute heart failure. Without intensive efforts to restrict blood volume, the same patients would have been considered to have been suffering from chronic heart failure, even though their underlying myocardial disease was no further advanced.

RIGHT-SIDED VERSUS LEFT-SIDED HEART FAILURE Many of the clinical manifestations of heart failure result from the accumulation of excess fluid behind either one or both ventricles (Chaps. 32 and 37). This fluid usually localizes upstream to (behind) the specific cardiac chamber that is initially affected. For example, patients in whom the left ventricle is mechanically overloaded (e.g., aortic stenosis) or weakened (e.g., postmyocardial infarction) develop dyspnea and orthopnea as a result of pulmonary congestion, a condition referred to as *left-sided heart failure*. In contrast, when the underlying abnormality affects the right ventricle primarily (e.g., valvular pulmonic stenosis or pulmonary hypertension secondary to pulmonary thromboembolism), symptoms resulting from pulmonary congestion such as orthopnea or paroxysmal nocturnal dyspnea are less common and edema, congestive hepatomegaly, and systemic venous distention, i.e., clinical manifestations of *right-sided heart failure*, are more prominent. However, when heart failure has existed for months or years,