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[1] NV Organon, Toxicology & Drug Disposition, Oss, The Netherlands
[2] University Medical Centre Nijmegen, Department of Pulmonary Diseases, Nijmegen, The Netherlands
[3] TNO Pharma, Biomolecular Sciences, Zeist, The Netherlands

Use of physicochemical calculation of pKa and CLogP to predict phospholipidosis-inducing potential

A case study with structurally related piperazines

JAN-PETER H. T. M. PLOEMEN[1], JAN KELDER[1], THEO HAFMANS[2], HAN VAN DE SANDT[3], JOHAN A. VAN BURGSTEDEN[3], PAUL J. M. SALEMINK[1], and ERIC VAN ESCH[1]

With 3 figures and 2 tables

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Address for correspondence: JAN-PETER H.T.M. PLOEMEN, NV Organon, Toxicology & Drug Disposition, PO Box 20, 5340 BH Oss, The Netherlands; Tel.: ++31-412 666 172, Fax: ++31-412 666131, e-mail: jan-peter.ploemen@organon.com

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Summary

Several cationic amphiphilic compounds are known to induce phospholipidosis, a condition primarily characterized by excessive accumulation of phospholipids in different cell types, giving the affected cells a finely foamy appearance. Excessive storage of lamellar membranous intralysosomal inclusion bodies is the hallmark for phospholipidosis on the electron microscopic level. In case of alveolar phospholipidosis, foamy macrophages accumulate within the alveolar spaces of the lung. Based on such findings in a one-year toxicity study with gepirone in rats, we studied the molecular properties of this compound and compounds suspected of being phospholipidosis inducers by means of physicochemical calculations. Physicochemical molecular calculations of molecular weight, ClogP (partition coefficient octanol/water), logD at pH 7.4, and pKa were performed, for the cationic amphiphilic compounds chlorpromazine, amiodarone, imipramine, propranolol and fluoxetine, and for the structurally related compounds 1-phenylpiperazine (1-PHP), gepirone (and its major metabolites, 3-OH-gepirone and 1-pyrimidinylpiperazine [1-PP]), and buspirone. ClogP and calculated pKa cluster differently for the amphiphilic drugs compared to the chemical series of piperazines. In line with this analysis, lamellar inclusion bodies were found in an in vitro validation experiment in the human monoblastoid cell line U-937, incubated for 96 h at 10 μg/mL with cationic amphiphilic drugs (amiodarone, imipramine, or propranolol). No such lamellar inclusion bodies were seen for any of the compounds from the chemical series of piperazines including gepirone and its metabolites. The data presented support the use of simple physicochemical calculations of ClogP and pKa to discriminate rapidly between compounds suspected of being phospholipidosis inducers. Finally, the discriminative power of these physicochemical ClogP and pKa calculations to predict phospholipidosis-inducing potential was further validated by extension of the set of compounds.

Introduction

Phospholipidosis is of concern for the pharmaceutical industry, since a candidate pharmaceutical agent may be rejected because of evidence of phospholipidosis in a preclinical animal study. Phospholipidosis is widely discussed and reported in rats (HALLIWEL 1997; GOPINATH et al. 1987; HRUBAN 1976; LÜLLMANN et al. 1975; REASOR 1989) and is characterized by the accumulation of phospholipids in the lysosomes of many cell types. Alveolar macrophages are especially susceptible to these changes, but other cell types may be affected as well, including lymphoid cells, hepatocytes, pancreatic cells, and cells within endocrine tissue, nervous system, muscle, and eyes. Phospholipidosis can be induced by the systemic administration of cationic amphiphilic drugs, like amiodarone, imipramine, or propranolol.

Alveolar histiocytosis is a common, spontaneous, incidental finding in older rats and consists of aggregates of foamy (lipid-containing) macrophages in the lumen of alveoli (BEAVER et al. 1963, BOORMAN and EUSTIS 1990;

Yang et al. 1966). The histopathological characteristics of this lesion have been discussed in many papers. Harmonization of nomenclature would be very welcome in this area, since many inconsistent morphologic descriptions, such as lipoproteinosis, pulmonary lipidosis and others are found in the literature (Boorman and Eustis 1990; Dungworth 1985; Gopinath et al. 1987; Kodavanti and Mehendale 1990). Alveolar phospholipidosis and alveolar histocytosis are both associated with the accumulation of intra-alveolar material, foamy macrophages and type II pneumocyte proliferation. The hallmark of phospholipidosis, multi-lamellar inclusion bodies ('whorls', 'myelin figures'), is easily detected using electron microscopy.

A one-year oral toxicology study was performed with gepirone, a new antidepressant presently under clinical development. Compared to the control group, an increased incidence of minimal to slight aggregations of foamy macrophages was observed: i.e. 1 animal in the control group, 1 animal in the low-dose (LD) group, 2 animals in the mid-dose (MD) group and 10 animals in the high-dose (HD) group. The lesion was evaluated in the context of the well-known, age-related spontaneous change in the rat, and judged as being of no relevance given that the increase in incidence occurred only at high multiples of the human dose. Subsequently, we performed a closer analysis of the physicochemical properties of gepirone due to the possibility that the higher incidence of alveolar foamy macrophages may be drug related. Physicochemical parameters of gepirone and its major metabolites were calculated and compared with well-known cationic amphiphilic drugs as a method for predicting phospholipidosis-inducing potential. Moreover, an in vitro study with human monoblastoid cell line U937 was performed to validate this method, using electron microscopic analysis.

Materials and methods

Reagents and media: RPMI 1640 culture medium, phosphate-buffered saline, Fetal Calf Serum (FCS) and penicillin/streptomycin were purchased from Invitrogen Corporation (UK). Chlorpromazine, amiodarone, imipramine, propranolol, 1-pyrimidinylpiperazine (1-PP) and 1-phenylpiperazine HCl (1-PHP) were obtained from from Sigma-Aldrich Chemical Corporation Ltd (Gillingham, UK); fluoxetine and buspirone from Tocris (Avonmouth, UK); and gepirone was obtained from Sidmak Laboratories Inc. (East Hanover, NJ, USA). 3-OH-gepirone was synthesized at Organon (Newhouse, Scotland) and was of >98% purity. All other chemicals were of analytical grade and were obtained from common commercial sources.

Animal experiment: A study was conducted to determine the toxicity of gepirone in Sprague-Dawley rats (weighing 147 to 215 g for the males and 117 to 176 g for the females and approximately 6 weeks at the start of dosing) when administered orally in the diet for 1 year. The study was conducted in 1987–1988 and was performed in compliance with OECD Principles of Good Laboratory Practice. Sprague-Dawley rats (22/sex/group) received 0, 4 (low-dose [LD]), 12 (mid-dose [MD], or 36 mg/kg/day (high-dose [HD]) in the diet. Each animal was individually housed in same humidity (40–60%) and controlled temperature 23 °C with a 12 hour light-dark cycle. A commercial diet (Purina Rodent Laboratory Chow #5001, supplied by Ralston Purina Co.) and tap water were available ad libitum. At week 19, the dose levels of 12 and 36 mg/kg/day were increased to 16 and 48 mg/kg/day in both male and female groups in order to achieve drug-related suppression of body weight gain or reduction in food intake. Mortality, clinical observations, body weights, and food and water consumption were recorded. Ophthalmoscopy was performed before dosing and during weeks 12, 26, and 52. Clinical (serum) chemistry determinations consisted of sodium, potassium, chloride, total protein, albumin, glucose, urea nitrogen, total cholesterol, alkaline phosphatase, alanine transaminase, total bilirubin, calcium, phosphorus, creatinine, creatine kinase, uric acid, triglycerides, aspartate transaminase, and lactate dehydrogenase at week 53 from the first 10 surviving rats (approximately fasted 18 hours) in each group. Hematology parameters (hematocrit, total hemoglobin, erythrocyte, reticulocyte, platelet, leukocyte, and leukocyte differential counts, plasma prothrombin and activated partial thromboplastin time) were measured at weeks 13, 27, and 52 from the first 10 surviving (nonfasted) rats in each group. Urinalysis samples (determinations made were gross appearance, volume, specific gravity, pH, protein, glucose, blood, bilirubin, urobilinogen, ketones, and microscopic findings) were analyzed at weeks 13, 27, and 51 on the first 5 animals in each group. Necropsy was performed on rats that died or were euthanized during the dosing period and on rats sacrificed at the end of the one-year treatment period. At necropsy, rats were examined macroscopically and selected organs were weighed. A complete histopathologic examination was performed on each animal. Lungs were fixed in buffered 10% formalin, paraffin embedded, and sections stained with hematoxylin and eosin. Below, we describe only issues relevant to the pulmonary foamy macrophages.

Molecular calculations: The static polar surface is calculated from the Corina-built structures using the in-house developed program called Monika (Kelder et al. 1999). Monika was also used to calculate molecular weight. The ClogP (partition coefficient octanol/water) was calculated with ClogP4.0 from BioByte Corporation, Claremont, CA, USA. LogD at pH 7.4 and pKa were calculated with ACD/Labs PhysChem Batch program release 7.0 (Advanced Chemistry Development, Inc., Toronto, Canada). For dibasic compounds, the lowest basic pKa >8 was used, and if not available, the highest pKa <8 was used. Gepirone, 3-OH-gepirone, 1-PP and buspirone belong to the same chemical series, as judged from the molecular structures. Calculation of ClogP and pKa were extended with a larger series of compounds known as 'positive' phospholipidosis inducers or negative controls.

Cell culture and drug incubations: A slightly adapted method as previously described by Xia et al. (1997) was used. The human monoblastoid cell line U-937 was cultured in RPMI 1640 culture medium containing heat-inactivated FCS (10%) penicillin (100 IU/mL) and streptomycin

(100 μg/mL) in 75 cm^2 flasks in a humidified incubator (37 ± 1 °C, 5% CO_2). Stock solutions of the drugs (1 mg/mL) were made in sterile phosphate-buffered saline. The cells (5×10^4/mL) were transferred from the flasks to 24 well plates (1 mL/well), and 10 μL of the stock solutions was added to the wells. All incubations were performed in duplicate. After a 48 h incubation, 0.5 mL of the culture medium (containing half of the cells) was removed from each well and 0.5 mL fresh medium was added. The number of cells was determined using a Bürker-Türk hemocytometer. After 96 h of incubation, the remaining cells were collected, counted and fixed for electron microscopic evaluation.

Sample preparation for electron microscopy: The cells were transferred to Eppendorf vials and washed two times in 0.15 M cacodylate buffer, pH 7.3 (4 °C). Subsequently, the cells were fixed in 1.5% glutaraldehyde in 0.15 M cacodylate buffer, pH 7.3 (4 °C) and stored at 4 °C. After fixation, cells were centrifuged and embedded in 1% agarose, rinsed in 0.1M phosphate buffer, osmicated for one hour in 1% osmiumtetroxide and rinsed again in phosphate buffer. Next, sections were dehydrated in a grade series of ethanol, embedded in epon 812 via propylene oxide. After polymerization, a Reichert Ultracut-E was used to cut 80 nm thick sections. The sections were examined with a Jeol 1010 electron microscope. Two independent observers examined and photographed only those cells that were sectioned through the region of the Golgi area and centrioles.

Results

Animal experiment

Dietary administration of gepirone to rats affected body weight in HD males and MD and HD females. Overall mean body weight gains for the male HD group and the female MD and HD groups were about 20–25% below that of the control group. Food consumption was increased in HD female rats. Focal pale areas were observed macroscopically in the lung in 1 control group male, 1 MD group female, 2 HD group males and 6 HD group females. With histopathological evaluation, minimal peribronchiolar lymphoid cellular infiltrates were located in the lungs from 32 control group, 34 LD group, 38 MD group, and 36 HD group rats. This pulmonary finding was considered to be a spontaneous or incidental observation. Histopathology revealed a slightly increased incidence of alveolar foamy macrophages in HD group males and females. Minimal to slight aggregations of foamy macrophages were observed in the alveolar lumens of 1 animal in the control group, 1 animal in the LD group, 2 animals in the MD group and 10 animals in the HD group. There was no relevant gender effect. In general, at the higher dose levels, the severity of the lesion did not increase but the foamy macrophages were more widespread throughout the lung. The body weight of animals with the lesion was, on average, approximately 24% lower than the body weight of control group animals of the same sex. Interestingly, animals with the foamy macrophages also weighed less than their counterparts (other animals of the same dose group not having foamy macrophages). This is illustrated by the fact that the animals with foamy macrophages had an average body weight approximately 13% lower than the average body weight of the corresponding group.

Comparison of the clinical chemistry, hematology, and urinalysis data with concurrent control values revealed several statistically significant differences which were minor, were within historical range, and were concluded to be of no toxicological significance.

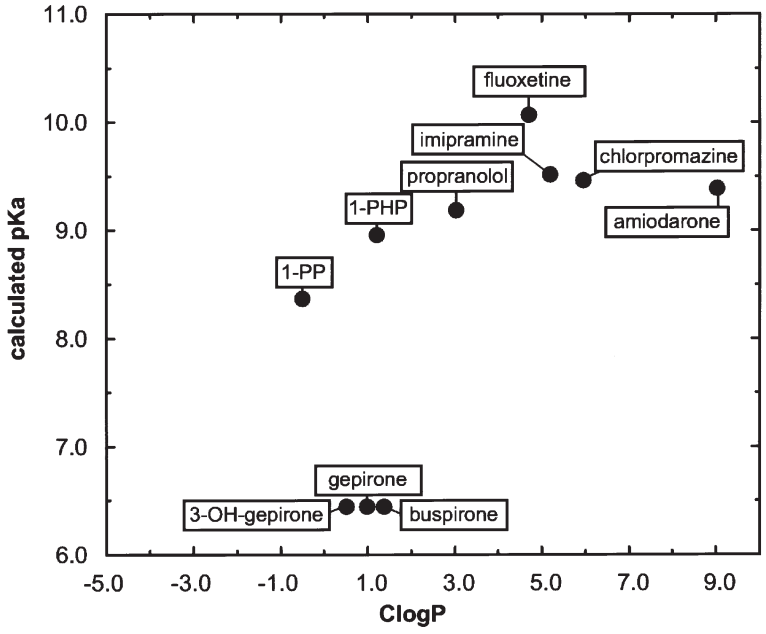
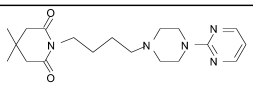
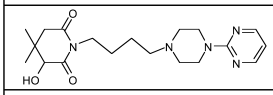
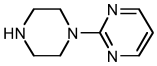
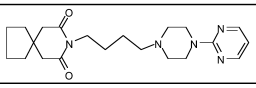
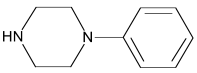
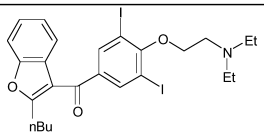
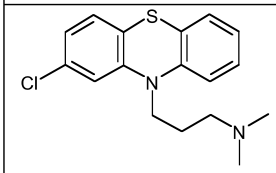
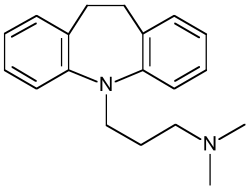
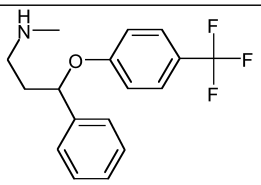
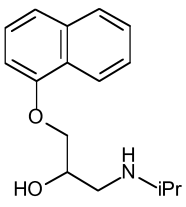


Fig. 1. Relation of Calculated ClogP and pKa of 10 drug molecules.

Table 1. Calculated physicochemical properties of 10 drug molecules.

Structure		MW	Pol Surface	ClogP	LogD (7.4)	Pka
Gepirone, its major metabolites, and chemically related compounds						
	Gepirone (Org 33062)	359.5	56.5	0.83	0.78	6.43
	3-OH-gepirone	375.5	72.8	0.40	0.36	6.42
	1-PP	164.2	34.0	-0.63	-1.62	8.34
	Buspirone	385.5	56.5	1.22	1.18	6.43
	1-PHP	162.2	14.0	1.08	-0.46	8.94
Cationic amphilic drugs						
	Amiodarone	645.3	34.9	8.95	6.99	9.37
	Chlorpromazine	318.9	8.0	5.80	3.80	9.43
	Imipramine	280.4	8.0	5.04	2.98	9.49
	Fluoxetine	309.3	18.8	4.57	2.04	10.05
	Propranolol	259.3	35.1	2.75	1.02	9.14

Molecular calculations

Physicochemical molecular calculations are summarized in table 1. With trend analysis, it appeared that a distinct cluster for amiodarone, chlorpromazine, imipramine, fluoxetine, and more or less propranolol was observed: cationic amphiphilic compounds combine a relatively high pKa with a high ClogP (fig. 1). The highest values of calculated pKa and ClogP were found as follows: amiodarone > chlorpromazine ~ imipramine ~ fluoxetine > propranolol. Unlike the series of cationic amphiphilic drugs, the chemical series of the structurally related piperazines showed different chemical properties. In the plot of the calculated ClogP versus pKa, gepirone, 3-OH-gepirone, and buspirone cluster differently than the cationic amphiphilic compounds (fig. 1). The 1-PP compound also had significantly lower pKa and ClogP values, and as predicted *a priori*, 1-PHP was closest to the predicted weakest amphiphilic compound (propranolol). To illustrate the discriminative power of the calculation of ClogP and pKa to differentiate phospholipidosis-inducing capacity for cationic amphiphilic compounds from negative controls, the calculations were extended with several known "negative controls" (i.e. diazepam, clozapine, 5-phenoxybenzamine, ketanserin, almitrine, haloperidol, bufetolol) and extra "positive controls" (fig. 2). Positive controls were divided in three categories i) positive controls with low phospholipidosis-inducing potency in animals, but pronounced potency to induce phospholipidosis in cultured cells: mianserin;

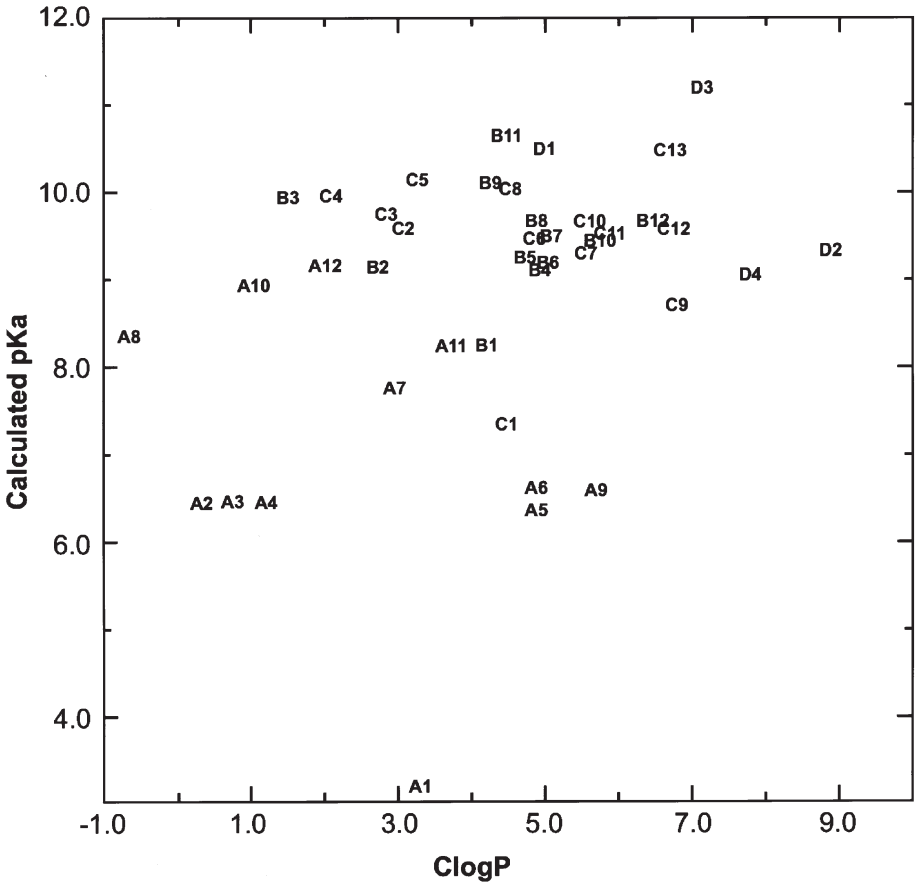


Fig. 2. Relation of Calculated ClogP and pKa of compounds positively or negatively associated with phospholipidosis inducing capacity. **A-series**; negative controls: A1, diazepam; A2, 3-OH-gepirone; A3, gepirone; A4, buspirone; A5, clozapine; A6, 5-phenoxybenzamine; A7, ketanserin; A8, 1-PP; A9, almitrine; A10, 1-PHP; A11, haloperidol; A12, bufetolol. **B-series**; positive controls with low phospholipidosis-inducing potency in animals, but pronounced potency to induce phospholipidosis in cultured cells: B1, mianserin; B2, propranolol; B3, clociguanil; B4, noxiptiline; B5, amitriptyline; B6, disobutamide; B7, promazine; B8, mesoridazine; B9, nortriptyline; B10, chlorpromazine; B11, maprotiline; B12, thioridazine. **C-series**; positive controls with phospholipidosis demonstrated in animals: C1, chlorcyclizine; C2, citalopram; C3, chlorphentermine; C4, phentermine; C5, fenfluramine; C6, imipramine; C7, tilorone; C8, fluoxetine; C9, tamoxifen; C10, iprindole; C11, clomipramine; C12, triparanol; C13, mepacrine. **D-series**; positive controls with phospholipidosis demonstrated in animals and in humans: D1, chloroquine; D2, amiodarone; D3, perhexiline; D4, desethylamiodarone. For dibasic compounds, the lowest basic pKa > 8 was used, and if not available, the highest pKa <8 was used. For compounds, chlorcyclizine (C1), mianserin (B1) and propranolol (B2) the reader is refered to the Discussion section. References are given in the Result section.

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