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[54] **GLUCAGON-LIKE PEPTIDE-2 ANALOGS**

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[58] **Field of Search** 514/2, 3, 12; 530/324, 530/303, 308; 935/52, 55, 66, 70, 71; 435/366, 371

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[57] **ABSTRACT**

Analogs of glucagon-like peptide 2, a product of glucagon gene expression, have been identified as intestinal tissue growth factors. Their formulation as pharmaceutical, and therapeutic use in treating disorders of the small bowel, are described.

23 Claims, No Drawings

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GLUCAGON-LIKE PEPTIDE-2 ANALOGS

This application is a continuation of application Ser. No. 08/631,273, filed Apr. 12, 1996, now abandoned, and a continuation-in-part of application Ser. No. 08/632,533, filed Apr. 12, 1996 and a continuation-in-part of Ser. No. 08/422,540, filed Apr. 14, 1995, the disclosures of which are incorporated by reference herein.

FIELD OF THE INVENTION

This invention relates to glucagon-related peptides having intestinal tissue growth promoting properties, and to their use therapeutically to treat various medical conditions resulting from the impaired growth or loss of such tissue.

BACKGROUND TO THE INVENTION

Expression of the glucagon gene yields a tissue-determined variety of peptide products that are processed from the 160 residue proglucagon product. The organization of these peptides within the proglucagon precursor was elucidated by the molecular cloning of preproglucagon cDNAs from the rat, hamster and bovine pancreas. These analyses revealed that preproglucagon contains not only the sequence of glucagon and glicentin, but also two additional glucagon-like peptides (GLP-1 and GLP-2) separated from glucagon and each other by two spacer or intervening peptides (IP-I and IP-II). These peptides are flanked by pairs of basic amino acids, characteristic of classic prohormone cleavage sites, suggesting they might be liberated after posttranslational processing of proglucagon (Drucker, Pancreas, V1990, 5(4):484).

Analysis of the peptides liberated from proglucagon in the pancreatic islets of Langerhans, for instance, suggests the primary pancreatic peptide liberated is the 29-mer glucagon, whereas glicentin, oxyntomodulin, IP-II and the glucagon-like peptides are more prevalent in the small and large intestines. This demonstration that the glucagon-like peptides are found in the bowel has prompted research into the precise structure and putative function(s) of these newly discovered gut peptides. Most studies have focussed on GLP-1, because several lines of evidence suggested that GLP-1 may be an important new regulatory peptide. Indeed, it has been determined that GLP-1 is one of the most potent known peptidergic stimulus for insulin release, an action mediated in a glucose-dependent manner through interaction with receptors on pancreatic β cells. GLP-1 and its derivatives are in development for use in the treatment of diabetics.

The physiological roles of glicentin and oxyntomodulin, the so-called "enteroglucagons", are also under investigation, particularly with respect to regulation of acid secretion and the growth of intestinal cells. Oxyntomodulin is capable of inhibiting pentagastrin-stimulated gastric acid secretion in a dose-dependent manner. The role of glicentin in mediating the changes of intestinal adaptation and growth of the intestinal mucosa has been investigated, and the intestinotrophic effect of glicentin and its therapeutic use have recently been reported by Matsuno et al in EP 612,531 published Aug. 31, 1994.

In contrast to GLP-1 and other glucagon-related peptides, the physiological role of glucagon-like peptide GLP-2 remains poorly understood despite the isolation and sequencing of various GLP-2 homologues including human, rat, bovine, porcine, guinea pig and hamster. Using GLP-2 antisera raised against synthetic versions of GLP-2, various groups have determined that GLP-2 is present primarily in intestinal rather than pancreatic extracts (see Mojsov et al, J. Biol. Chem., 1986, 261(25):11880; Orskov et al in Endocrinology, 1986, 119(4):1467 and in Diabetologia, 1987, 30:874 and in FEBS Letters, 1989, 247(2):193; George et al, FEBS Letters, 1985, 192(2):275). With respect to its biological role, Hoosein et al report (FEBS Letters, 1984, 178(1):83) that GLP-2 neither competes with glucagon for binding to rat liver and brain tissues, nor stimulates adenylate cyclase production in liver plasma membranes, but, enigmatically, can stimulate adenylate cyclase in both rat hyopthalamic and pituitary tissue, at 30–50 pM concentrations. An elucidation of the physiological role of GLP-2 would clearly be desirable.

SUMMARY OF THE INVENTION

There have now been discovered analogs of GLP-2 which promote growth of small bowel tissue. It is accordingly a general object of the present invention to provide such GLP-2 analogs and to provide for their use therapeutically and for related purposes.

In one aspect of the invention, the GLP-2-analogs exhibit intestinotrophic activity and conform to the structural Formula 1 (SEQ ID NO:1):

R1-(Y1)m-X1-X2-X3-X4-Ser5-Phe6-Ser7-Asp8-(P1)-Leu14-Asp15-Asn16-Leu17-Ala18-X19-X20-Asp21-Phe22-(P2)-Trp25-Leu26-Ile27-Gln-28-Thr29-Lys30-(P3)-(Y2)n-R2,

wherein

X1 is His or Tyr

X2 is Ala or an Ala-replacement amino acid conferring on said analog resistance to DPP-IV enzyme;

X3 is Asp or Glu;

X4 is Gly or Ala;

Pi is Glu-X10-Asn-Thr-Ile or Tyr-Ser-Lys-Tyr (SEQ ID NO:3);

X10 is Met or an oxidatively stable Met-replacement amino acid;

X19 is Ala or Thr;

X20 is Arg, Lys, His or Ala;

P2 is Ile-Asn, Ile-Ala or Val-Gln;

P3 is a covalent bond, or is Ile, Ile-Thr or Ile-Thr-Asp;

R1 is H or an N-terminal blocking group;

R2 is OH or a C-terminal blocking group;

Y1 is one or two basic amino acids selected from the group Arg, Lys, and His;

Y2 is one or two basic amino acids selected from the group Arg, Lys, and His; and

m and n, independently, are 0 or 1; and

wherein at least one of X1, X2, X3, X4, P1, X10, X19, X20, P2 and P3 is other than a wild type, mammalian GLP-2 residue.

Particularly preferred analogs according to Formula 1 are those which are rendered resistant to cleavage by human DPP-IV enzyme by replacing the Ala at position X2 with an alternative amino acid. Other analogs of the invention are those which replace the oxidatively sensitive Met at position X10 with an amino acid residue which is oxidatively stable. In this manner, the analog peptides have increased stability compared to GLP-2 peptides with the wild-type Met residue at this position. Yet another preferred embodiment of the invention is the incorporation at position X20 of a basic amino acid selected from His or Lys. This substitution is advantageous when the GLP-2 analogs are chemically syn-

thesized. The Arg residue which normally occurs at this position tends to strongly bind solvents used in peptide synthesis procedures. Substitution of the Arg allows easier formulation of the synthetically produced GLP-2 analogs into pharmaceutically acceptable compositions.

More particularly, and according to one aspect of the invention, there are provided analogs of a GLP-2 peptide selected from a mammalian GLP-2 species and N- and/or C-terminally modified forms thereof, the analogs having intestinotrophic activity and incorporating, relative to said mammalian GLP-2 peptide, at least one amino acid substitution at a position which is conserved in mammalian GLP-2's. In a preferred aspect, the GLP-2 analogs incorporate a substitution selected from:

1) incorporation at position 2 or at position 3 of an Ala replacement amino acid conferring on said analog resistance to Dipeptidyl Peptidase-IV (hereinafter referred to as DPP-IV); and
2) incorporation at position 10 of an oxidatively stable Met-replacement amino acid; and
3) incorporation at X20 of a replacement amino acid other than Arg.

In another of its aspects, the invention provides a pharmaceutical composition comprising a GLP-2 analog of the present invention in a therapeutically effective amount, and preferably in an intestinotrophic amount, and a pharmaceutically acceptable carrier.

In a further aspect, the invention provides a method for promoting growth of small bowel tissue in a patient in need thereof, comprising the step of delivering to the patient an intestinotrophic amount of a GLP-2 analog of the present invention.

Besides promoting bowel growth, in another of its aspects the invention provides a method for treating a gastrointestinal disease by administering to a patient suffering from gastrointestinal disease a therapeutically effective amount of a GLP-2 analog of the invention, together with a pharmaceutically acceptable carrier, in order to reduce a pathological effect or symptom of the gastrointestinal disease.

In still another aspect of the invention, there is provided a method useful to identify intestinotrophic analogs of GLP-2, comprising the steps of:

1) obtaining a GLP-2 analog conforming to Formula 1 represented above;
2) treating a mammal with said analog using a regimen capable of eliciting an intestinotrophic effect when utilized for rat GLP-2; and
3) determining the effect of said analog on small bowel weight relative to a mock treated control mammal, whereby said intestinotrophic analog of GLP-2 is identified as an analog which elicits an increase in said weight.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to therapeutic and related uses of GLP-2 analogs, particularly for promoting growth of tissue of the small bowel. The effect on growth elicited by the present GLP-2 analogs manifests as an increase in small bowel weight, relative to a mock-treated control. In particular, GLP-2 analogs are considered to have "intestinotrophic" activity if, when assessed in the murine model exemplified herein, the analog mediates an increase in small bowel weight of at least 10% relative to a control animal receiving vehicle alone. Particularly suitable for therapeutic use are those analogs which mediate an increase of at least 20% in small bowel weight; preferred for therapeutic use are those which mediate an increase in small bowel weight of 50% or more. Intestinotrophic activity is noted most significantly in relation to the jejunum, including the distal jejunum and particularly the proximal jejunum, and are also noted in the ileum.

In addition to exhibiting intestinotrophic activity as just defined, the GLP-2 analogs of the present invention incorporate an amino acid substitution at one or more sites within a GLP-2 peptide "background", which is either a mammalian GLP-2 species per se, or is a variant of a mammalian GLP-2 species in which the C-terminus and/or the N-terminus has been altered by addition of one or two basic residues, or has been modified to incorporate a blocking group of the type used conventionally in the art of peptide chemistry to protect peptide termini from undesired biochemical attack and degradation in vivo. Thus, the present peptides incorporate an amino acid substitution in the context of any mammalian GLP-2 species, including but not limited to human GLP-2, bovine GLP-2, rat GLP-2, degu GLP-2, ox GLP-2, porcine GLP-2, guinea pig GLP-2 and hamster GLP-2, the sequences of which have been reported by many authors, including Buhl et al, J. Biol. Chem., 1988, 263(18):8621.

In one aspect of the invention, the intestinotrophic analogs of GLP-2 conform to the sequence of Formula 1 (SEQ ID NO:1) as follows:

R1-(Y1)m-X1-X2-X3-X4-Ser5-Phe6-Ser7-Asp8-(P1)-Leu14-Asp15-Asn16-Leu17-Ala18-X19-X20-Asp21-Phe22-(P2)-Trp25-Leu26-Ile27-Gln-28-Thr29-Lys30-(P3)-(Y2)n-R2,

wherein

X1 is His or Tyr

X2 is Ala or an Ala-replacement amino acid conferring on said analog resistance to DPP-IV enzyme;

X3 is Asp or Glu;

X4 is Gly or Ala;

P1 is Glu-X10-Asn-Thr-Ile or Tyr-Ser-Lys-Tyr (SEQ ID NO:3);

X10 is Met or an oxidatively stable Met-replacement amino acid;

X19 is Ala or Thr;

X20 is Arg, Lys, His or Ala;

P2 is Ile-Asn, Ile-Ala or Val-Gln;

P3 is a covalent bond, or is Ile, Ile-Thr or Ile-Thr-Asp;

R1 is H or an N-terminal blocking group;

R2 is OH or a C-terminal blocking group;

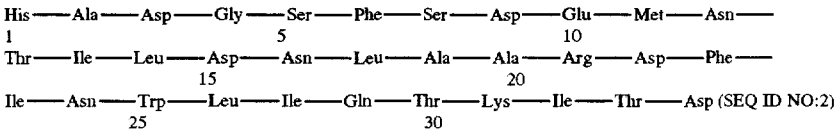
Y1 is one or two basic amino acids selected from the group Arg, Lys, and His;

Y2 is one or two basic amino acids selected from the group Arg, Lys, and His; and

m and n, independently, are 0 or 1; and

wherein at least one of X1, X2, X3, X4, P1, X10, X19, X20, P2 and P3 is other than a wild type, mammalian GLP-2 residue.

Wild-type mammalian GLP-2 residues which occur at a specific position are determined by aligning the sequences of GLP-2's isolated from different mammalian species and comparing the sequence to the human sequence, reproduced below, for convenience (SEQ ID NO:2):



The amino acid residues which, for purposes of this application, are known to occur at specific positions in wild type mammalian GLP-2's are the following: position X13 may be Ile or Val; Position X16 may be Asn or Ser; position X19 may be Alanine or Threonine; position X20 may be Arg or Lys; position X27 may be Ile or Leu; and position X28 may be Gln or His.

The present GLP-2 analogs may incorporate desired amino acid substitutions into a "background" which is an N-terminally or C-terminally modified form of a mammalian GLP-2 peptide. Such analogs are represented in Formula 1 as those in which R1 constitutes an N-terminal blocking group, and/or when m is 1 then Y1 is one or two basic amino acids such as Arg or Lys; and/or R2 is a C-terminal blocking group; and/or when n is 1 then Y2 is independently, one or two basic amino acids such as Arg or Lys.

In preferred embodiments of the invention, the GLP-2 analog is an analog of full length GLP-2, i.e., GLP-2(1–33), and P3 is accordingly the sequence Ile-Thr-Asn. Alternatively, the GLP-2 analogs may be C-terminally truncated, to yield GLP-2(1–32) forms in which P3 is Ile-Thr, or GLP-2(1–31) forms in which P3 is Ile, or GLP-2(1–30) forms in which P3 is a covalent bond.

The "blocking groups" represented by R1 and R2 are chemical groups that are routinely used in the art of peptide chemistry to confer biochemical stability and resistance to digestion by exopeptidase. Suitable N-terminal protecting groups include, for example, C_{1-5}alkanoyl groups such as acetyl. Also suitable as N-terminal protecting groups are amino acid analogues lacking the amino function. Suitable C-terminal protecting groups include groups which form ketones or amides at the carbon atom of the C-terminal carboxyl, or groups which form esters at the oxygen atom of the carboxyl. Ketone and ester-forming groups include alkyl groups, particularly branched or unbranched C_{1-5}alkyl groups, e.g., methyl, ethyl and propyl groups, while amide-forming groups include amino functions such as primary amine, or alkylamino functions, e.g., mono-C_{1-5}alkylamino and di-C_{1-5}alkylamino groups such as methylamino, ethylamino, dimethylamino, diethylamino, methylethylamino and the like. Amino acid analogues are also suitable for protecting the C-terminal end of the present compounds, for example, decarboxylated amino acid analogues such as agmatine.

Embodiments of the invention specifically include such analogs in which m is 0 and R1 is a blocking group such as acetyl; and analogs in which m is 0 and R2 is a C-terminal blocking group such as an amine, e.g., —NH2.

In a preferred aspect of the invention, the GLP-2 analogs are analogs of either human GLP-2 or of rat GLP-2. Human GLP-2 is herein referred to interchangably as hGLP-2 (1–33). Rat GLP-2 has the amino acid sequence of human GLP-2, but incorporates at position 19 a Thr residue instead of an Ala residue. Rat GLP-2 is accordingly referenced herein either as rGLP-2(1–33) or as the Thr^{19} analog of human GLP-2, i.e., as [Thr^{19}]hGLP-2(1–33).

In particularly preferred embodiments of the invention, with respect to both the Formula 1 analogs and the more specific human or rat GLP-2 analogs, the GLP-2 analogs incorporate an amino acid substitution selected from:

1) incorporation at X2 and/or at X3 of a replacement amino acid which renders said analog resistant to cleavage by DPP-IV enzyme;
2) incorporation at X10 of an oxidatively stable Met-replacement amino acid; and
3) incorporation at X20 of a replacement amino acid other than Arg.

The DPP-IV-resistant class of GLP-2 analogs possess particularly advantageous properties. As is demonstrated herein, mammalian GLP-2 species have been found to be sensitive to cleavage by DPP-IV enzyme. It has also been found that this sensitivity to DPP-IV is the result of the recognition sequence Ala^2Asp^3found in all mammalian forms of GLP-2. There are accordingly provided by the present invention a class of GLP-2 analogs which incorporate at X2 and/or X3 a replacement amino acid which confers on the GLP-2 analog relative resistance to DPP-IV mediated cleavage, as determined by any convenient in vitro or in vivo assessment technique that is able to detect the presence of GLP-2 digestion products. A DPP-IV resistant GLP-2 analog is revealed as that GLP-2 analog which is processed or degraded at a rate that is measurably slower than the rate at which human GLP-2 is processed or degraded, under the same conditions.

An assay suitable for assessing DPP-IV sensitivity and resistance is described below in Example 3, in the context of results actually obtained.

The X2 class of GLP-2 analogs is preferred herein. These Ala^2-substituted GLP-2 analogs can incorporate at X2 a structurally wide variety of Ala-replacement amino acids to achieve relative resistance to DPP-IV digestion. A similarly wide variety of Ala-replacement amino acids allow also for the retention by the analog of intestinotrophic activity. For purposes of identifying those DPP-IV-resistant X2 analogs that also retain intestinotrophic activity, the X2 analogs showing DPP-IV resistance are screened in the assay of intestinotrophic activity described below in Example 4.

In embodiments of the present invention, the Ala^2 replacements include stereoisomers of amino isomers that would otherwise be substrates for DPP-IV, for example D-Ala, D-HPr and D-Pro; naturally occurring amino acids other than Ala, HPr and Pro which provide a basic or uncharged side chain, for example, Glu, Lys, Arg, Leu, Ile, Gly and Val. In specific embodiments of the invention, there are provided the following Ala^2-substituted GLP-2 analogs: [D-Ala^2]rGLP-2(1–33), [Gly^2]rGLP-2(1–33), [Val^2]rGLP-2 (1–33) and [Gly^2]hGLP-2(1–33).

The X2 GLP-2 analogs may incorporate amino acid replacements at other positions. In embodiments of the invention, such analogs include those carrying amino acid substitutions also at one or more of positions X1, X3, X4, X10, X19, X20 and X24, and therefore include those which, according to Formula 1, include at least one of the following substitutions: X1 is Tyr; X3 is Glu; X4 is Ala; P1 is Glu-X10-Asn-Thr-Ile where X10 is other than Met or P1 is Tyr-Ser-Lys-Tyr; X10 is an oxidatively stable Met-replacement amino acid; X19 is Thr; X20 is Lys or Ala; P2 is Val-Gln and P3 is a covalent bond, Ile, or Ile-Thr or Ile-Thr-Asn.

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