Novel Long-Acting Somatostatin Analog with Endocrine Selectivity: Potent Suppression of Growth Hormone But Not of Insulin

MICHEL AFARGAN, EVA TIENSSU JANSON, GARRY GELERMAN, RAKEFET ROSENFELD, OFFER ZIV, OLGA KARPOV, AMNON WOLF, MOSHE BRACHA, DVIRA SHOHAT, GEORGE LIAPAKIS, CHAIM GILON, AMNON HOFFMAN, DAVID STEPHENSKY, AND KJELL OBERG

Departments of Medicinal Sciences and Endocrine Oncology (E.T.J., K.O.), University Hospital SE 75185, Uppsala, Sweden; Department of Organic Chemistry, Faculty of Life Sciences (C.G.), and Department of Pharmaceutical Sciences, School of Pharmacy, Faculty of Medicine (A.H., D.H.), Hebrew University, Jerusalem 91904, Israel; Department of Pharmacology, Medical School, University of Crete (G.L.), Heraklion, Crete 71110, Greece; and Peptor Ltd., Kiryat Weizmann, Rehovot 76326, Israel

ABSTRACT

Somatostatin, also known as somatotropin release-inhibiting factor (SRIF), is a natural cyclic peptide inhibitor of pituitary, pancreatic, and gastrointestinal secretion. Its long-acting analogs are in clinical use for treatment of various endocrine syndromes and gastrointestinal anomalies. These analogs are more potent inhibitors of the endocrine release of GH, glucagon, and insulin than the native SRIF; hence, they do not display considerable physiological selectivity. Our goal was to design effective and physiologically selective SRIF analogs with potential therapeutic value. We employed an integrated approach consisting of screening of backbone cyclic peptide libraries constructed on the basis of molecular modeling of known SRIF agonists and of high throughput receptor binding assays with each of the five cloned human SRIF receptors (hsst1–5). By using this approach, we identified a novel, high affinity, enzymatically stable, and long-acting SRIF analog, PTR-3173, which binds with nanomolar affinity to human SRIF receptors hsst2, hsst4, and hsst5. The hsst5 and the rat sst5 (rsst5) forms have the same nanomolar affinity for this analog. In the human carcinoid-derived cell line BON-1, PTR-3173 inhibits forskolin-stimulated cAMP accumulation as efficiently as the drug octreotide, indicating its agonistic effect in this human cell system. In hormone secretion studies with rats, we found that PTR-3173 is 1000-fold and more than 10,000-fold more potent in inhibiting GH release than glucagon and insulin release, respectively. These results suggest that PTR-3173 is the first highly selective somatostatinergic analog for the in vivo inhibition of GH secretion, with minimal or no effect on glucagon and insulin release, respectively. (Endocrinology 142: 477–486, 2001)

SOMATOSTATIN (SRIF), a peptide hormone originally isolated from the hypothalamus as a GH-releasing inhibiting factor, has been found throughout the central nervous system as well as in widely distributed endocrine and exocrine cells in the periphery (1, 2). The hormone acts on a diverse array of endocrine, exocrine, neuronal, and immune cell targets to inhibit secretion, modulate neurotransmission, and regulate cell division. Physiologically, SRIF has a potent inhibitory effect on the secretion of a large number of hormones, including GH, insulin, glucagon, gastrin, cholecystokinin, and other mediators secreted by the pituitary, pancreas, and the gastrointestinal tract (1–4). These biological functions are mediated via high affinity interaction of SRIF with a family of six cell receptors. These receptors, named sst1, -2A, -2B, -3, -4, and -5, are encoded by five genes and belong to the G protein-coupled receptor family. The two different sst2 forms are product of a common gene and are generated by alternative splicing, with sst2A being the unspliced and sst2B being the spliced product of the sst2A messenger RNA (3–5). The human and rodent forms of sst1, sst2, asst3, or sst4 are known to have similar binding properties. However, the sequence of sst5 is the most divergent, with only 81% identity between the human and rat sequences, and there are significant differences in the affinities of these two forms for various octapeptide analogs of SRIF (3, 4). Functionally, SRIF receptors seem to be linked to different signal transduction pathways, including adenylate cyclase, ion conductors, and protein dephosphorylation. Over the past few years, the five receptor genes have been cloned and characterized (3–7). Antibody probes have been developed for each of the SRIF receptors and have been useful in indicating their potential function (8). In the pituitary gland, somatotrophs have predominant expression of sst2 and sst5 receptors, indicating that both receptor subtypes may have a role in regulation of GH secretion (3–10). The sst1 and sst5 receptors have been strongly colocalized with insulin in the pancreatic β-cells, and pancreatic α-cells are rich in sst2 (9–11).

The native SRIF, which exists in two major forms, a tetradecapeptide (SRIF-14) and a 28-amino acid form (SRIF-28), is readily proteolized by aminopeptidases and endopeptidases and has a short in vivo half-life of about 2–3 min. Synthetic SRIF analogs were developed for clinical applications. These share with the native SRIF its pharmacophore,
the essential amino acid sequence Phe$^7$-Trp$^8$-Lys$^9$-Thr$^{10}$ (the numbering follows that of native SRIF) responsible for efficacy, and are metabolically stabilized at both N- and C- terminals (12). To date, three commercially available cyclic (disulfide bridged) SRIF analogs: octreotide (SMS201995; d-Phe-Cys-Phe-d-Trp-Lys-Thr-Cys-Thr(ol)), lanreotide (BIM 23014; d-Penal-Cys-Tyr-d-Trp-Lys-Val-Cys-Thr-NH$_2$), and vapreotide (RC160; d-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Trp-NH$_2$) have been shown to be clinically effective in various endocrine and gastrointestinal abnormalities (for review, see Ref. 13). These SRIF analogs all have similar binding affinities for four of the five human SRIF receptor subtypes (hsst): high affinity for hsst2 and hsst5, moderate affinity for hsst3, and very low affinity for hsst1. Lanreotide and vapreotide have a moderate affinity for hsst4, whereas octreotide has little or no affinity for this human SRIF receptor (13–15). These drugs are long acting, with circulating half-lives of about 90 min; however, their clinical use is limited, because they lack considerable endocrine selectivity (14, 15). This family of drugs inhibits with high potency the endocrine release of GH, glucagon, and insulin compared with the native SRIF (3, 13–19).

In humans, long-term treatment with SRIF analogs is sometimes associated with hyperglycemia due to their inhibitory effects on insulin secretion (16, 17, 19–21). In this report we present the in vitro and in vivo evaluations of a novel SRIF analog, PTR-3173, which was identified by using an integrated drug discovery approach. The prime objective of this program was to select ligands from the backbone cyclic (22–24) SRIF series possessing in vivo endocrine efficacy and selectivity.

Materials and Methods

All reagents used for in vitro and in vivo studies were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Chemistry

Libraries of backbone cyclic SRIF analogs tested in this study were synthesized by solid phase, multiple parallel synthesis, using F-moc chemistry as previously described (23), which was adapted to a 96-well format. Synthesis was performed on an ACT 396 synthesizer (Advanced ChemTech, Louisville, KY) equipped with a Lab Tech 4 (Advanced ChemTech, Louisville, KY) for heating. The backbone cyclic building units were protected on their ω-carboxy by allyl/alloc protecting group, which was removed before on resin cyclization. The synthesis scale was selected for the synthesis of 100 mg of product. The synthesis scale was selected for the synthesis of 100 mg of product.

Radioligand binding assay

Ligand binding assays were performed with membranes isolated from CHO-K1 cells expressing the cloned human sst receptors hsst1, -2, -3, and -5; the rat sst5; COS-7 cells expressing hsst-4 receptor; and the human carcinoid cell line BON-1 (a gift from Prof. J. C. Thompson, Galveston, TX), as previously described (25, 26). The ligand $^{[35]}$S-Tyr$^3$-somatostatin-14 (0.05 μCi; SA, 2000 Ci/mmol; Amersham Pharmacia Biotech, Little Chalfont, UK) was used at a final concentration of 0.1 nM. Assays were performed in 96-well polystyrene microtiter plates (Maxisorp plates, Nunc, Copenhagen, Denmark) in a final volume of 250 μl. The assay buffer consisted of 50 mM Tris-HCl (pH 7.8), 1 mM EGTA, 5 mM MgCl$_2$, leupeptin (10 μg/ml), bacitracin (200 μg/ml), aprotinin (0.5 μg/ml), and phenylmethylsulfonylfluoride (0.1 mM). At the end of the binding reaction, free radioligand was separated from bound ligand by a rapid filtration through UniFilter GF/C plates filters (Whatman, Clifton, NJ) treated with 0.5% polyethyleneimine and 0.1% BSA. The filters were performed on a FilterMate Cell Harvester (Packard Instrument Co., Downers Grove, IL). Plates were washed with cold 50 mM Tris-HCl (pH 7.8), then dried before counting in a TopCount Microplate Scintillation Counter (Packard Instrument Co., Downers Grove, IL). Ligand binding assays were performed in triplicate wells and repeated three times.

Screening cycles

Backbone cyclic SRIF libraries were screened against the five cloned human SRIF receptors (hsst1, -2, -3, -4, and -5) in ligand binding assays, which were adapted to a 96-well format. The screening approach consisted of three cycles. The first and second cycles were performed with crude samples, and the third was performed after HPLC purification. Samples were tested for binding with each of the receptors at a dilution of 1,000-fold, which resulted in an estimated final concentration in the micromolar range. Samples that displaced over 50% of the radioligand were tested again at a dilution of 10,000-fold (estimated final concentration per well in the nanomolar range). Samples that displaced over 50% of the radioligand at this concentration were purified and restested over a range of concentrations between 0.1–10,000 nM.

cAMP assay

cAMP was tested in the human pancreatic carcinoid cell line, BON-1, that expresses SRIF receptors (27–29). Cells were grown in DMEM (AppliChem GmbH, Darmstadt, Germany) and F12K nutrient mixture, Kattgäns Modified (Life Technologies, Inc.), at a ratio of 1:1 containing 5% FCS. The BON-1 cells were preincubated in medium containing 200 μM isobutyryl-1-methylxanthine (ICN Biomedicals, Inc., Costa Mesa, CA) and subsequently exposed to 10 μM forskolin (ICN Biomedicals, Inc.) with or without test substances for 30 min at 37°C. The cultures were extracted in ice-cold propanol (Labscant Ltd., Analytical Sciences, Shannon, Ireland) and after evaporation were assayed for cAMP by RIA (NEN Life Science Products, Brussels, Belgium).

Metabolic stability

The metabolic stability of PTR-3173 was determined by incubation of the pure analogs with various tissue enzyme mixtures as previously described (30, 31). Briefly, peptides were incubated at a final concentration of 0.1 mg/ml in human serum, rat renal homogenate, or rat liver homogenate for up to 4 h at 37°C. Samples were withdrawn at several time points, and the percentage of the unchanged molecules was analyzed by HPLC. Elimination of unstable peptide was verified by the apparent reduction of the area under the curve of the chromatogram major peak. Degradation was confirmed by newly emerging peaks (i.e. fragments) that were derived from the peptide compared with the blank chromatogram as a control.

ELISA

Development of a three-step competition ELISA enabled monitoring of PTR-3173 concentrations in body fluids for the assessment of its pharmacokinetics and pharmacodynamics. Briefly, specific polyclonal antibodies (Abs) were purified from rabbit serum after three immunizations with PTR-3173 conjugated to keyhole limpet hemocyanin. Immunoaffinity purification of Abs was performed on a protein G-Sepha-
rose 4 Fast Flow chromatography column (Pharmacia Biotech, Uppsala, Sweden) with PTR-3173 bound to BSA. Secondary Abs of goat antirabbit conjugated to alkaline phosphatase were used for detection. The limit of detection was about 10 ng/ml PTR-3173. Specificity for PTR-3173 was confirmed by the lack of recognition of native SRIF and other SRIF synthetic analogs.

In vivo experiments

All animal procedures were reviewed and approved by the national committee for ethical animal care and use in Israel. Male Wistar rats (Harlan Sprague Dawley, Inc., Jerusalem, Israel) were used for pharmacokinetic and pharmacodynamic studies. Rats were obtained at the age of 6–7 weeks (mean weight, 200 ± 20 g) and were acclimated for a period of at least 1 week before experiments.

Pharmacodynamics

The pharmacodynamics of SRIF analogs were determined in hormone release assays with rats as previously described (31–33). Briefly, animals were fasted for 16–18 h before experiments. Endocrine stimulation was performed under Nembutal anesthesia (60 mg/kg, ip). Stimulation of GH and glucagon was induced by iv administration (through the femoral vein) of l-arginine (0.5 g/kg). Insulin release was tested after an iv bolus administration of d-glucose (0.5 g/kg). At 5 min after hormone stimulation, blood was collected from the abdominal vena cava. Plasma hormone levels were measured using commercial RIA kits. Rat GH was determined by BIOTRAK RIA (Amersham Pharmacia Biotech; catalog no. RPA551). Rat glucagon and insulin were determined by RIA kits obtained from Linco Research, Inc. (St. Louis, MO; catalog no. GL-32K for glucagon and catalog no. RI-13K for insulin). All drugs were dissolved in isotonic acetic (pH 4.0) buffer and administered sc before hormone stimulation. In each experiment rats were segregated into four main groups (n = 10–15) according to drug treatment: PTR-3173, octreotide, control (acetic acid buffer, pH 4.0), and untreated (without stimulation). ED₅₀ suppression values of GH, glucagon, and insulin release were measured at 15 and 30 min after drug administrations.

Pharmacokinetics

Pharmacokinetic studies were performed with conscious rats using jugular vein cannulation (PE-50, Intramedic, Becton Dickinson and Co., Sparks, MD) for blood collection, as previously described (33). The cannulation was performed under anesthesia 48 h before the day of the experiment to allow full recovery of the animals from the surgical procedure. Two modes of administration were tested for PTR-3173 pharmacokinetics: iv and sc. Animals received an iv bolus dose of 0.5 mg/kg PTR-3173 dissolved in isotonic acetic acid buffer (pH 4.0), or a sc dose of 1 mg/kg PTR-3173 dissolved in the same buffer. Blood samples (with heparin, 15 U/ml) were collected at time intervals of up to 24 h after PTR-3173 administration. Plasma and urinary concentrations of PTR-3173 were measured by PTR-3173 enzyme-linked immunosorbent assay. Half-life (t₁/₂), volume of distribution (Vss), and clearance (CL) of PTR-3173 were calculated using WinNonlin software, standard edition version 1.1 (Scientific Consulting, Inc., Cary, NC).

Data analysis

All results are expressed as the mean ± sem. The comparisons between treatment groups were analyzed by one-way ANOVA for repeated measures at the 95% confidence level. When significant overall effects were obtained by ANOVA, multiple comparisons were made using Dunnett’s test. P < 0.05 was considered statistically significant.

Results

Lead discovery approach: design of libraries of backbone cyclic SRIF analogs

The chemical structure of SRIF is depicted in Fig. 1. Numerous SRIF analogs have been synthesized and studied, resulting in the identification of the key residues essential for binding and biological activity. These residues, Phe⁶-Phe⁷-Trp⁸-Lys⁹-Thr¹⁰-Phe¹¹ (the numbering follows that of native SRIF) were used to construct smaller analogs displaying SRIF activity. The central structure of Fig. 1 depicts the chemical structure of the drug octreotide, in which the phenylalanine residues at positions 6 and 11 were replaced by two cysteine residues to enable cyclization. The numerous small SRIF analogs that have been synthesized have enabled the identification of a smaller, sequential pharmacophore Phe/Tyr/Trp²⁸-Lys²⁹-Thr³⁶-Phe³⁷ (the numbering follows that of native SRIF) necessary for biological activity. This sequence has been shown to adapt a
type II β-turn in all active analogs. Figure 2 depicts the pharmacophore sequence in the well studied analog L363, 301 (also called the Veber compound) (12).

The backbone cyclization technology enables the creation of libraries that comprise a large number of conformationally constrained peptidic analogs by bridging any two positions along their backbone through bridges of varying sizes and chemical compositions. Thus, in an attempt to identify novel

**FIG. 4.** A, Displacement of $[^{125}\text{I}]$Tyr$^{11}$-SRIF-14 binding by PTR-3173 to CHO-K1 cells transfected with hst1 (□), hst2 (■), hst3 (▲), and hst5 (●) and to COS-7 cells transfected with hst4 (●) receptor clones. Assays were performed with membranes isolated from the transfected cells. The ligand $[^{125}\text{I}]$Tyr$^{11}$-SRIF-14 was used at a final concentration of 0.1 nM. The unlabeled PTR-3173 was examined over a range of concentrations from 0.1–1000 nM. B, Comparison of displacement of $[^{125}\text{I}]$Tyr$^{11}$-SRIF-14 binding by PTR-3173 to human (●) and rat (○) sst5 receptor subtype clones.

*Fig. 3.* Distribution of binding affinities of diversified backbone cyclic SRIF analogs to cloned human SRIF receptors. A, The first cycle of screening was performed with crude peptides formed and samples diluted by 1,000-fold and tested for their affinities to hst3 and hst5. B, Binding results of the second round with the same receptors. Only active samples that possess more than 50% displacement in the first round were tested after 10,000-fold dilution. The estimated peptide concentration was in the nanomolar range. C, Binding affinities of several samples that possess hst2 and hst5 specificity; 1,000-fold diluted samples (○) and 10,000-fold diluted samples (●) are shown. The red dot represents the high affinity of PTR-3173 to these human SRIF receptors.
SRIF analogs, we designed libraries of compounds with identical or highly similar sequences to both of the above pharmacophoric sequences (the six- and four-residue pharmacophores) that are entrapped in various conformations, with the limitation that all bridges would enable the formation of the β-turn around the pharmacophore. Four such libraries, containing 96 compounds each, were synthesized and screened.

The results of the screening cycles are shown in Fig. 3. In Fig. 3A are shown the results of screening against hsst3 and -5 at a 1,000-fold dilution (estimated concentration of peptides in the micromolar range) of crude samples. Samples displaying more than 50% inhibition at this dilution were retested at a 10,000-fold dilution (nanomolar range concentrations; Fig. 3B). Figure 3C depicts the results of screening against receptors hsst2 and -5 at both dilutions. Screening of the libraries resulted in the identification of various single receptor-selective analogs (data not shown) as well as analogs that selectively bind to various receptor combinations. Various reports in the literature have demonstrated that hsst2-selective analogs do not show pharmacological selectivity (3, 17), specifically they do not discriminate between physiological effect mediated by SRIF analogs, we designed libraries of compounds with identical or highly similar sequences to both of the above pharmacophores that are entrapped in various conformations, with the limitation that all bridges would enable the formation of the β-turn around the pharmacophore. Four such libraries, containing 96 compounds each, were synthesized and screened.

The results of the screening cycles are shown in Fig. 3. In Fig. 3A are shown the results of screening against hsst3 and -5 at a 1,000-fold dilution (estimated concentration of peptides in the micromolar range) of crude samples. Samples displaying more than 50% inhibition at this dilution were retested at a 10,000-fold dilution (nanomolar range concentrations; Fig. 3B). Figure 3C depicts the results of screening against receptors hsst2 and -5 at both dilutions. Screening of the libraries resulted in the identification of various single receptor-selective analogs (data not shown) as well as analogs that selectively bind to various receptor combinations. Various reports in the literature have demonstrated that hsst2-selective analogs do not show pharmacological selectivity (3, 17), specifically they do not discriminate between the inhibitory effects on the secretion of GH and insulin. We observed similar results with the backbone cyclic hsst2-selective analogs (data not shown). This led us to hypothesize that selectivity to unique receptor combinations, rather than single receptor subtype selectivity, could dissociate the physiological effect mediated by SRIF analogs. Indeed, PTR-3173 (Figs. 1 and 3) was shown in our screening process to posses a unique binding profile, binding with high affinity to receptor subtypes hsst2, -4, and -5. It was therefore chosen as a candidate for further examination.

**In vitro studies with PTR-3173**

**PTR-3173 binds with high affinity to a novel combination of human SRIF receptors.** After the discovery of the unique binding profile of PTR-3173 we synthesized the compound in a large scale, followed by HPLC purification. Figure 4a shows that in CHO-K1 cells, PTR-3173 displaced the binding of [125I]Tyr11-SRIF to the cloned receptors hsst2 and hsst5 with IC50 values of 3 and 6 nM, respectively. In COS-7 cells expressing the cloned receptor subtype hsst4, PTR-3173 showed an IC50 of 7 nM. PTR-3173 recognized with very low affinity hsst1 (>1000 nM) and hsst3 (>100 nM). As among the ss1–5 receptor subtypes a relatively low homology exists between human and rat ssst5 forms (3, 4), we tested the binding affinities of rat cloned ssst5 for PTR-3173. Figure 4b shows the comparison of receptor binding between human and rat ssst5 cloned receptors for PTR-3173. PTR-3173 has an IC50 of 8 nM to the rat ssst5 form, virtually equipotent to that to the cloned human ssst5 receptor.

**PTR-3173 receptors displayed agonistic inhibition of adenylyl cyclase in human BON-1 cells.** SRIF receptors were previously shown to mediate the inhibition of forskolin-stimulated cAMP accumulation in various cell systems known to express SRIF receptors (3–6). We tested the effects of PTR-3173 and octreotide in comparison to native SRIF on forskolin-stimulated cAMP accumulation in the human carcinoid-derived BON-1 cells. BON-1 cells were previously shown to express SRIF receptors (27–29) and serve as an in vitro assay for the examinations of carcinoid therapeutics (28, 29). To examine whether the binding of PTR-3173 to human SRIF receptors mediates agonistic or antagonistic activity we tested its effects in native human cell system rather than in the transfected CHO cells, because the human BON-1 cell system is more applicable to the clinic. Before the cAMP assay with BON-1, we examined whether PTR-3173 recognized SRIF receptors expressed in BON-1. Figure 5 shows the displacement curves of [125I]Tyr11-SRIF from isolated BON-1 membranes by the unlabeled ligands: SRIF, PTR-3173, and octreotide. The IC50 values reveal that SRIF binds to BON-1 with an IC50 of 2 nM, whereas under the same experimental conditions PTR-3173 and octreotide displaced the binding of [125I]Tyr11-SRIF with IC50 values of 18 and 32 nM, respectively. The relatively lower IC50 values of these ligands compared to that displayed by SRIF were in accordance with the relative abundance in these cells of the receptors they recognize (29). Figure 6 shows the effects of SRIF, PTR-3173, and octreotide on forskolin-stimulated cAMP accumulation in...
BON-1 cells. SRIF inhibited cAMP accumulation by 50% with a potency of 10 nM. The SRIF analogs octreotide and PTR-3173 also inhibited adenylyl cyclase activity in this cell line, suggesting that each of these compounds displays agonistic effects mediated by the human SRIF receptors expressed in BON-1 cells.

PTR-3173 exhibits interfamily specificity to the SRIF receptor family. The interfamily cross-talk between G protein-coupled receptors is a well known phenomenon (34–38). As it has been shown that SRIF and its synthetic analogs interact with other G protein-coupled receptors, such as the opiate and neurokinin (NK) receptors (35, 37–40), and as PTR-3173 was chosen for further study due to its unique receptor binding profile, we tested whether PTR-3173 recognized other G protein-coupled receptors. Figure 7 shows the displacement data of specific radioligands by SRIF, PTR-3173, and octreotide from opiate, NK, and muscarinic receptors. Neither native SRIF nor the two analogs show significant inhibition of NK1 and NK3 binding (Fig. 7A). Octreotide, but neither the native hormone nor PTR-3173, inhibits binding to the muscarinic M2 receptor (Fig. 7A). All three compounds display binding inhibition of the opiate receptors (Fig. 7A), yet at physiological concentrations (Fig. 7B) only octreotide shows significant inhibition (~80% at 100 nM). All three compounds recognized human recombinant NK2 receptors (Fig. 7A), but none of them inhibited binding to this receptor at physiological concentrations (Fig. 7B).

PTR-3173 exhibits metabolic stability to degradation by enzymes. To confirm the metabolic stability of PTR-3173 before its administration to animals the analog was subjected to a series of degradative enzymes. The results demonstrate that PTR-3173 is a highly stable analog, comparable in its stability to the metabolically stable drug octreotide. Both compounds showed a significantly higher metabolic stability in human serum (Fig. 8) than the native hormone SRIF-14. Similar results were obtained in rat renal homogenate (data not shown).

In vivo studies with PTR-3173

The pharmacokinetics of PTR-3173 in the rat. The in vitro metabolic stability studies of PTR-3173 suggests that this compound is expected to have a long half-life in the circulation. Figure 9 and Table 1 show the pharmacokinetics of PTR-3173 after iv bolus or sc administration to conscious rats. The plasma concentrations of PTR-3173 show biexponential characteristics after iv bolus administration (Fig. 9). These results demonstrate that the pharmacokinetics of PTR-3173 are similar to those demonstrated for octreotide in rats, as reported by Sandoz Pharmaceuticals Corp. (Hannover, NJ) (42). Approximately the same volumes of distribution and clearance were observed for both drugs (Table 1).

A considerable pharmacokinetic value of PTR-3173 was found after sc administration. The elimination of PTR-3173 from the systemic circulation in the postabsorptive phase was significantly slower than in the case of iv bolus administration (Fig. 9). The bioavailability after the sc administra-
tion was calculated to be 100%. The elimination half-life was about 170 min, indicating its slow absorption rate from the injection site. Approximately 16% of the sc dose was excreted in the urine as unchanged PTR-3173. In the blood, PTR-3173 as well as octreotide were distributed mainly in the plasma. Approximately 66% of PTR-3173 was bound to plasma proteins.

Effect of PTR-3173 on GH, glucagon, and insulin release in Wistar rats.

After confirmation of the metabolic stability and pharmacokinetics of PTR-3173, we tested its pharmacodynamic effect on hormone release in Wistar rats. We used the drug octreotide as a positive control rather the native SRIF in the animal studies, because octreotide has a long duration of action in vivo and its pharmacodynamics are well documented in the literature. The efficacy results with PTR-3173 and octreotide are shown here adapted from Ref. 42.

Table 1. Pharmacokinetic constants of PTR-3173 and octreotide in rats

<table>
<thead>
<tr>
<th>Route</th>
<th>Drug</th>
<th>F (%)</th>
<th>Vss (ml/kg)</th>
<th>t1/2 (min)</th>
<th>% Excreted in urine</th>
<th>(CL) (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iv</td>
<td>PTR-3173</td>
<td>653</td>
<td>31</td>
<td>10.3</td>
<td>13.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Octreotide</td>
<td>602</td>
<td>49</td>
<td>21.3</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td>sc</td>
<td>PTR-3173</td>
<td>99.6</td>
<td>170</td>
<td>15.9</td>
<td>13.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Octreotide</td>
<td>103</td>
<td>40</td>
<td>23.0</td>
<td>17.1</td>
<td></td>
</tr>
</tbody>
</table>

F, Bioavailability; Vss, volume of distribution; T1/2, circulating half-life; CL, clearance. The pharmacokinetic values of octreotide presented here adapted from Ref. 42.

Effect of PTR-3173 on GH, glucagon, and insulin release in Wistar rats. After confirmation of the metabolic stability and pharmacokinetics of PTR-3173, we tested its pharmacodynamic effect on hormone release in Wistar rats. We used the drug octreotide as a positive control rather than the native SRIF in the animal studies, because octreotide has a long duration of action in vivo and its pharmacodynamics are well documented in the literature. The efficacy results with PTR-3173 and octreotide are shown in Fig. 10. Drugs were administered sc with a fixed dose of 100 μg/kg, which is above the reported ED50 of octreotide for inhibition of GH (0.1 μg/kg), glucagon (0.65 μg/kg), or insulin (26 μg/kg) release (Table 2) (31). Figure 10A shows the suppression of L-arginine-stimulated GH release by PTR-3173 compared with the effect of octreotide. PTR-3173 and octreotide are equipotent inhibitors of GH release under the same experimental conditions. Figure 10B shows the plasma glucagon levels found in the same blood samples used for GH measurements. PTR-3173 significantly reduced glucagon release, but with a lower potency than octreotide. Figure 10C shows the effects of PTR-3173 and octreotide on d-glucose-induced insulin release. Octreotide significantly decreased insulin levels compared with control values, consistent with previous reports (21, 42). Under the same experimental conditions PTR-3173 did not affect insulin release when administered at 100 μg/kg or at a 10-fold higher dose of 1 mg/kg.

Dose-response relationship of GH and glucagon release by PTR 3173

After the first line in vivo studies with PTR-3173 we performed dose-response relationship studies of GH and glucagon release. Figure 11 shows that although PTR-3173 inhibited GH release with an ED50 of 0.1 μg/kg similar to octreotide, glucagon release was inhibited only at 100 μg/kg. Thus, PTR-3173 is a 1,000-fold more potent selective inhibitor of GH than of glucagon release, whereas octreotide is less selective for GH, with a GH/glucagon potency ratio of about 8 (Table 2) (31). The selectivity of PTR-3173 is even more dramatic for insulin vs. GH; PTR-3173 had practically no inhibitory effect on insulin secretion (ED50 >1,000 μg/kg); therefore, its relative selectivity for GH over insulin was more than 10,000-fold, whereas this ratio for octreotide is 309.

Discussion

In this report we describe a novel high affinity ligand, PTR-3173, of the human SRIF receptors. Physiologically, PTR-3173 is equipotent to other long-acting SRIF analogs in the inhibition of GH release. However, in contrast to the clinically available SRIF analogs that are also potent inhibitors of glucagon and insulin, PTR-3173 is 1,000-fold more potent in the in vivo inhibition of GH than of glucagon and does not affect insulin secretion at physiological concentrations (GH/insulin potency ratio, >10,000). This is the first description of a long-acting SRIF analog possessing complete in vivo selectivity between GH and insulin inhibition. Thus,
The inhibition of the release of insulin and its counter-regulatory hormones glucagon and GH by SRIF is well documented (1–3, 13). There are six subtypes of SRIF receptor, which are differentially expressed in various endocrine and exocrine organs (3–6). Much effort has been directed toward identification of the exact physiological roles of each of the SRIF receptor subtypes. Despite the extensive data accumulated on the subject, no definitive results have yet been obtained. Several synthetic agonists with in vivo receptor subtype binding selectivity greater than 100-fold have recently become available for each of the hsst receptors (25). However, in vivo studies revealed that none of these analogs is able to separate between GH and insulin secretion inhibition (25, 43).

We observed similar in vivo results (data not shown) with various single receptor subtype-specific backbone cyclic SRIF analogs. Consequently, it led us to the assumption that specific receptor-profiles rather than singlereceptor subtype specificity could dissociate the physiological effects mediated by SRIF or its analogs.

Our data suggest that the unique receptor binding combination of PTR-3173 is associated with a significant selectivity of GH vs. insulin secretion inhibition in vivo. PTR-3173 recognizes with high affinity (nanomolar range) a novel receptor subset combination, hsst2, hsst4, and hsst5, and does not recognize the opiate receptor. Compounds showing high affinity to hsst2, hsst3, and hsst5 have been reported by others, yet none of the known synthetic SRIF analogs displays high affinity to hsst4 (3, 4, 13).

In the cAMP assay in the human carcinoid-derived cell line BON-1, PTR-3173 was shown to be an agonist of SRIF receptors, similar to what has been found with the drug octreotide. However, as BON-1 cells express a heterologous pattern of SRIF receptors (27–29), it is not possible to determine which subtype of hsst receptor in this cell system mediated the apparent net agonistic effect of PTR-3173. The problem of defining the exact receptor-effector relationship is also relevant for the physiological effects displayed by PTR-3173 in the hormone release assays. Although the potent inhibitory effect of PTR-3173 on GH release supports its agonistic effect as a SRIF analog in vivo, we were unable to determine which of its receptors or which receptor combination mediates this effect. Recent pharmacological studies in rats have suggested that the ssst2 receptor subtype mediates SRIF inhibition of GH and glucagon (25, 43). As the affinities of human and rat ssst5 receptors toward SRIF (17). As the affinities of human and rat ssst5 receptors toward SRIF are known to differ (for instance, hsst5 has a 160-fold lower affinity for octreotide than the rat receptor) (3, 4), interpretation of our in vivo studies performed with rats required an evaluation of the affinity for the rat ssst5. The results clearly demonstrate that the unique physiological selectivity possessed by PTR-3173 in rats is not due to different binding affinities for rat and human receptors. Therefore, the question of whether ssst2, ssst5, or a combination of these receptors plays the principal role in insulin secretion in the rat has yet to be determined.

It is suggested that the unique pharmacology of PTR-3173 may provide a new pharmacotherapy approach for various endocrine abnormalities where the endocrine nonselective SRIF analogs are not efficient.

**FIG. 10.** The pharmacodynamics of PTR-3173 compared with octreotide were evaluated by hormone release assays performed with Wistar rats under Nembutal anesthesia. Plasma GH and glucagon levels were measured in the same blood samples, which were collected from fasted animals at 5 min after iv administration of L-arginine. Plasma insulin levels were measured by a separate experiment; blood was collected 5 min after L-glucose was administered iv. Octreotide or PTR-3173 was administered sc at 15 min before blood collection with an equal dose of 100 μg/kg. A, Plasma GH levels at 5 min after L-arginine administration to control animals (□) compared with the nonstimulated animals (untreated) and those given PTR-3173 (■) and octreotide (●). Both analogs suppressed with equal potency GH release by more than 95% compared with control levels. B, Plasma glucagon levels were measured under the same experimental conditions of GH analysis. Octreotide significantly inhibited glucagon release by 70%, whereas PTR-3173 reduced plasma glucagon levels with less potency (55%) than octreotide compared with control values. C, Plasma insulin levels were reduced significantly in the octreotide-treated group. The reported ED<sub>50</sub> of octreotide of insulin release was 26 μg/kg (31), which was confirmed by our study. PTR-3173, at 100 or 1000 μg/kg, did not affect insulin release. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (vs. control).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control n=10</th>
<th>Untreated n=10</th>
<th>Octreotide 100 μg/kg n=15</th>
<th>PTR-3173 1000 μg/kg n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma GH Level (ng/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octreotide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTR-3173</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasma Glucagon Level (ng/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octreotide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTR-3173</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasma Insulin Level (ng/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octreotide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTR-3173</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A NOVEL, PHYSIOLOGICALLY SELECTIVE SOMATOSTATIN ANALOG

Tables and diagrams:

**TABLE 2.** Pharmacodynamic constants: inhibitory effects of PTR-3173 and octreotide on GH, glucagon, and insulin secretions

<table>
<thead>
<tr>
<th>Effect</th>
<th>GH ED$_{50}$ (µg/kg)</th>
<th>Glucagon ED$_{50}$ (µg/kg)</th>
<th>Insulin ED$_{50}$ (µg/kg)</th>
<th>Potency ratio GH/insulin</th>
<th>Potency ratio GH/glucagon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octreotide</td>
<td>0.08</td>
<td>0.65</td>
<td>26</td>
<td>309</td>
<td>8</td>
</tr>
<tr>
<td>PTR-3173</td>
<td>0.1</td>
<td>100</td>
<td>&gt;1,000</td>
<td>&gt;10,000</td>
<td>1,000</td>
</tr>
</tbody>
</table>

The data presented here for octreotide were adapted from Ref. 31. PTR-3173 and octreotide are equipotent inhibitors of GH secretion. PTR-3173 is a 1,000-fold more potent selective inhibitor of GH than of glucagon and in a more than 10,000-fold more potent inhibitor of GH than of insulin.

**Figure 11.** Dose-response relationships of PTR-3173 on L-arginine-induced GH and glucagon release in Wistar rats. ED$_{50}$ values were measured using the experimental protocols of hormone release described in Materials and Methods. PTR-3173 reduced plasma GH levels with ED$_{50}$ of 80–100 ng/kg after 15 (●) and 30 (■) min of drug administration. The dose-response studies with glucagon (△) release reveals that at 15 min after sc administration, 100 µg/kg PTR-3173 was the only dose that produced significant inhibition (50%; P < 0.05) of glucagon secretion. *, P < 0.05; **, P < 0.01; ***, P < 0.01 (vs. control).

Although the mechanism governing the in vivo selectivity of PTR-3173 is unclear, we suggest three possibilities: 1) a direct effect of activating the receptor subset sst2, sst4, and sst5 present in the rat pituitary and the endocrine pancreas (3, 5, 8, 10, 41, 42); 2) lack of PTR-3173 binding to the opiate receptors; and 3) differential activation of sstr2 subtypes A and B by PTR-3173.

Taking together the nonselectivity of sst2-specific analogs with possibility 1 suggests that sst4 may act as a counter-regulatory receptor. Possibility 2 is reasonable in light of the data demonstrating high affinity binding of octreotide to the opiate receptor. The interaction between the opiate receptor and insulin secretion has been described previously (40, 46). As for possibility 3, differential expression patterns for the two forms of the sst2 receptor have been reported (8, 11, 41) in the pancreas. Although the ligand binding characteristics of these variants are expected to be identical, it cannot be ruled out that their activation can be ligand dependant (3, 8).

Thus, differential activation of these variants by PTR-3173 could also be responsible for the separation of its effects on the secretion of GH and insulin.

To investigate the assumption of a counterregulatory receptor responsible for the observed selectivity, we examined the in vivo effect on insulin release using a pharmacodynamic interaction approach. We coadministered high doses of PTR-3173 with either octreotide or an hsst2-selective backbone cyclic analog, both displaying potent in vivo inhibition of insulin in our models. We assumed that if PTR-3173 activates a possible counterregulatory receptor responsible for the lack of activity on insulin secretion, it could affect the inhibitory effect of these compounds as well. The results of this study (data not shown) revealed that PTR-3173 does not significantly affect the inhibition of insulin release mediated by octreotide or by our hsst2-selective ligand. Thus, the effect of PTR-3173 in vivo is not caused by a counterregulatory SRIF receptor. Further research is necessary to test whether other mechanisms mediated by the unique sst receptor binding profile of PTR-3173 are responsible for its selectivity, or whether this is caused by lack of binding to the opiate receptors or to sst2 variant-specific binding. Studies to address this question could be performed using receptor variant-specific antibodies.

Recent evidence suggests that the GH-insulin-like growth factor I axis could play a principal role in the pathophysiology of diabetic and other nephropathies (47, 48). Our observed endocrine selectivity suggests that PTR-3173 may be useful for treating diabetes and diabetic complications such as nephropathy (47, 48). Phillip et al. (submitted for publication) evaluated the effect of PTR-3173 on experimental diabetic nephropathy in the nonobese diabetic (NOD) mice. In these studies, repetitive sc administrations of PTR-3173 at 1 mg/kg/day for 21 days caused a significant reduction of renal/glomerular hypertrophy, reduced creatinine hyperfiltration, and had a blunting effect on serum GH levels in the diabetic animals. Consistent with the lack of effect of PTR-3173 on insulin and glucagon secretion, serum glucose levels were not altered by this treatment. Combining these pharmacological characteristics with its therapeutic potential, it is suggested that PTR-3173 may have clinical utility for the treatment of various endocrine abnormalities associated with increased activity of the GH-insulin-like growth factor I axis. The unique binding of PTR-3173 to receptor subtype 2, 4, and 5 and its various biological activities might be useful for treatment of metabolic diseases such as diabetes type 2 and acromegaly.

**References**

A NOVEL, PHYSIOLOGICALLY SELECTIVE SOMATOSTATIN ANALOG

Endo • 2001 Vol. 142 • No. 1


44. Rosowskii WJ, Coy DH 2005 Specific inhibition of rat pancreatic insulin or glucagon secretion by receptor-selective somatostatin analogs. Biochem Biophys Res Commun 334:346

