

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

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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 somatostatinergetic 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, sst3, 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 conduction channels, 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 proteolyzed 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,

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the essential amino acid sequence Phe⁷-Trp⁸-Lys⁹-Thr¹⁰ (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-βNal-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH₂), and vapreotide (RC160; D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH₂) 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 6 μmol Rink amide MBHA resin, which resulted in approximately 5 mg crude product/well. Amino acid coupling was carried out with F-moc-protected amino acids using HBTU/HOBT and diisopropylethyl amine in *N*-methylpyrrolidinone at room temperature. Coupling to the *N*-alkylated amino acid moieties, which were used for cyclization, was performed with bistrichloromethylcarbonate and collidine in dioxane and 1,3-dichloropropane at 50 C (23). F-moc groups were removed by 25% piperidine in *N*-methylpyrrolidinone. At the end of the assembly allyl/alloc deprotection on the bridging arms was performed with tetrakis (triphenylphosphine)-palladium (0). Cyclization was performed on the resin using benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate. Cleavage from the resin was carried out by a reagent mixture (92.5% trifluoroacetic acid, 2.5% triisopropyl silane, and 2.5% ethane dithiol, 1 h). The raw peptides were partially purified by C₁₈ Sep-Pak chromatography using 50% acetonitrile in water as eluent. The molecular weight of the expected peptides was determined by mass spectroscopy. Large scale synthesis was carried out according to procedures described previously (23, 24).

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 [¹²⁵I]Tyr¹¹-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 polypropylene 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 MgCl₂, leupeptin (10 μg/ml), bacitracin (200 μg/ml), aprotinin (0.5 μg/ml), and phenylmethylsulfonyl fluoride (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% polyethylenimine and 0.1% BSA. The filtration was 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). The 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 retested 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, Kaighn's Modification (Life Technologies, Inc.), at a ratio of 1:1 containing 5% FCS. The BON-1 cells were preincubated in medium containing 200 μM isobutyl-L-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 (Labsan 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 0.4 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-

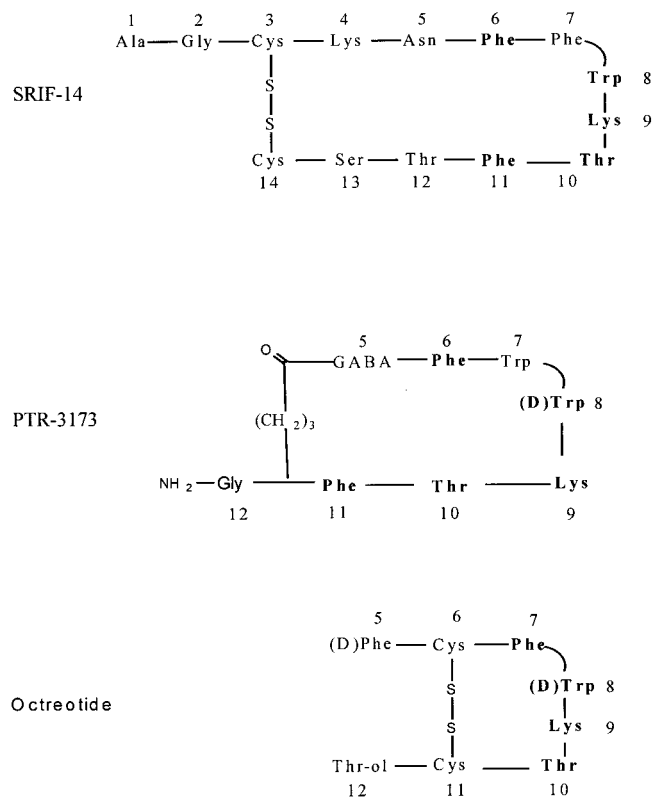


FIG. 1. Amino acid compositions of SRIF, octreotide, and PTR-3173. Amino acids that share with the native SRIF its suggested pharmacophore are shown in **bold**.

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

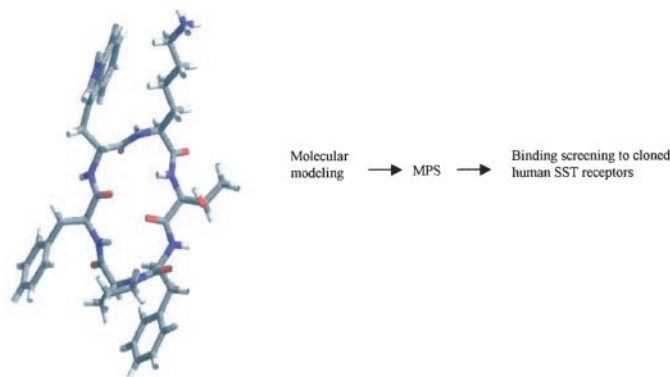


FIG. 2. Libraries constructed on the basis of the sequential pharmacophore model were synthesized by the multiple parallel synthesis technique adapted to 96 format and were subjected to biological screening to the five separate binding assays with each of the cloned human SRIF receptors.

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 (t_{1/2}), volume of distribution (V_{ss}), 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/Trp⁷-D-Trp⁸-Lys⁹-Thr/Val¹⁰ (3, 12, 24, 31) necessary for biological activity. This sequence has been shown to adapt a

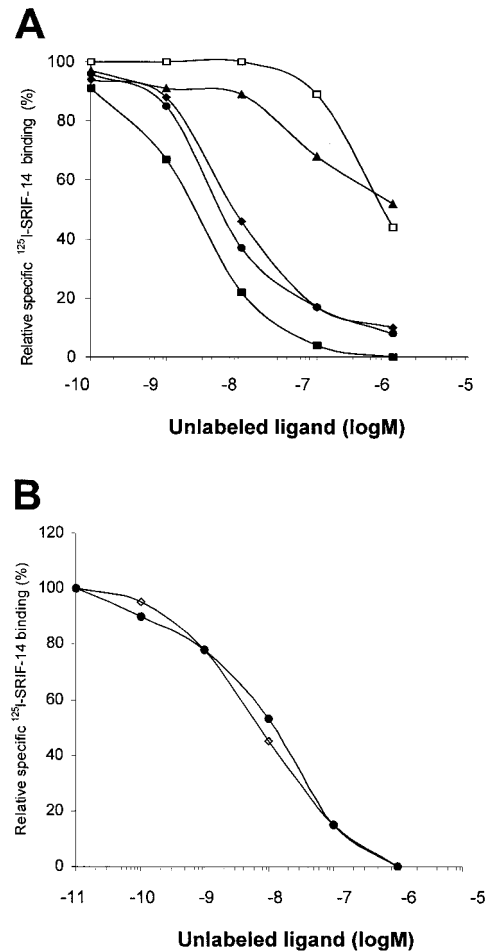
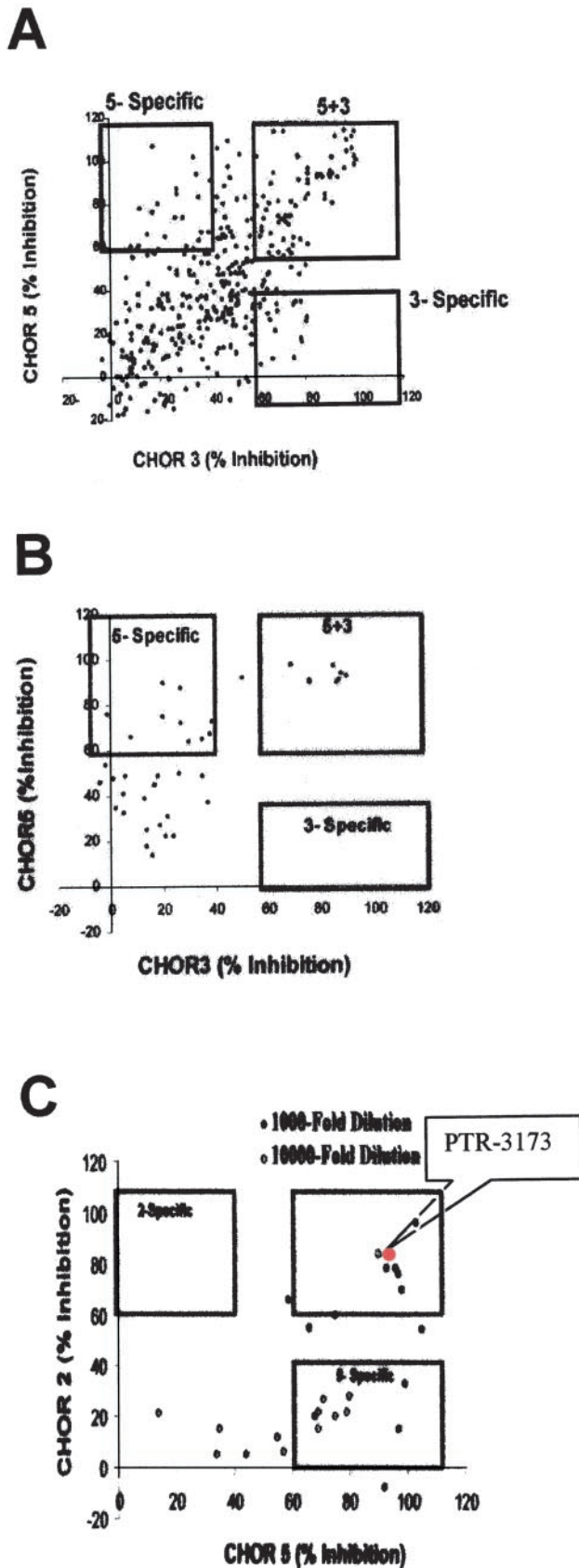


FIG. 4. A, Displacement of [125 I]Tyr 11 -SRIF-14 binding by PTR-3173 to CHO-K1 cells transfected with hsst1 (\square), hsst2 (\blacksquare), hsst3 (\blacktriangle), and hsst5 (\bullet) and to COS-7 cells transfected with hsst4 (\blacklozenge) receptor clones. Assays were performed with membranes isolated from the transfected cells. The ligand [125 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 I]Tyr 11 -SRIF-14 binding by PTR-3173 to human (\bullet) and rat (\diamond) sst5 receptor subtype clones.

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. 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 hsst3 and hsst5. 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 hsst2 and hsst5 specificity; 1,000-fold diluted samples (\circ) and 10,000-fold diluted samples (\bullet) are shown. The red dot represents the high affinity of PTR-3173 to these human SRIF receptors.

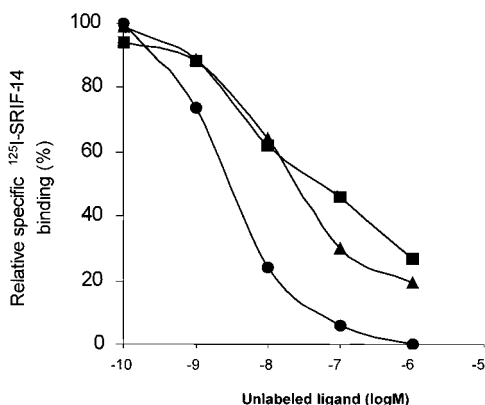


FIG. 5. Displacement of [125 I]Tyr 11 -SRIF-14 binding to human carcinoid-derived BON-1 cells by unlabeled ligands (●, SRIF; ■, octreotide; ▲, PTR-3173). Membrane fractions isolated from cells were used for binding studies as described in *Materials and Methods*. Displacement of [125 I]Tyr 11 -SRIF-14 (0.1 nM) by the unlabeled ligands was examined over a range of concentrations (0.1–1000 nM).

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 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 possess 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

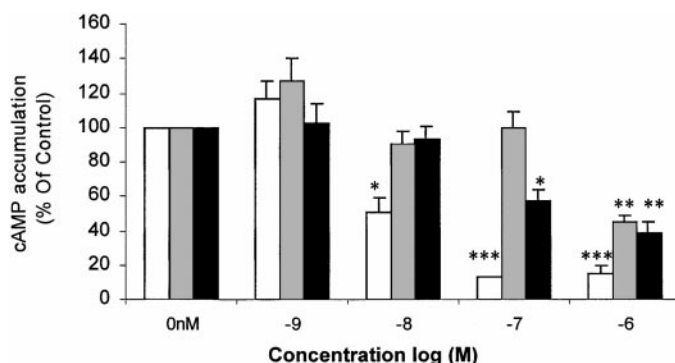


FIG. 6. Concentration-dependent inhibition of forskolin-stimulated cAMP accumulation in BON-1 cells by SRIF (□), octreotide (■) and PTR-3173 (■). Cells were untreated (control, bar 0) or treated with increasing concentrations of the test substances for 30 min. Results are expressed as a percentage of the control value with untreated cells (mean \pm SEM of five separate experiments performed in duplicate). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (vs. control).

[125 I]Tyr 11 -SRIF to the cloned receptors hsst2 and hsst5 with IC_{50} values of 3 and 6 nM, respectively. In COS-7 cells expressing the cloned receptor subtype hsst4, PTR-3173 showed an IC_{50} of 7 nM. PTR-3173 recognized with very low affinity hsst1 (>1000 nM) and hsst3 (>100 nM). As among the sst1–5 receptor subtypes a relatively low homology exists between human and rat sst5 forms (3, 4), we tested the binding affinities of rat cloned sst5 for PTR-3173. Figure 4b shows the comparison of receptor binding between human and rat sst5 cloned receptors for PTR-3173. PTR-3173 has an IC_{50} of 8 nM to the rat sst5 form, virtually equipotent to that to the cloned human sst5 receptor.

PTR-3173 receptors displayed agonistic inhibition of adenylate 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 (10 μ M)-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 [125 I]Tyr 11 -SRIF from isolated BON-1 membranes by the unlabeled ligands: SRIF, PTR-3173, and octreotide. The IC_{50} values reveal that SRIF binds to BON-1 with an IC_{50} of 2 nM, whereas under the same experimental conditions PTR-3173 and octreotide displaced the binding of [125 I]Tyr 11 -SRIF with IC_{50} values of 18 and 32 nM, respectively. The relatively lower IC_{50} 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

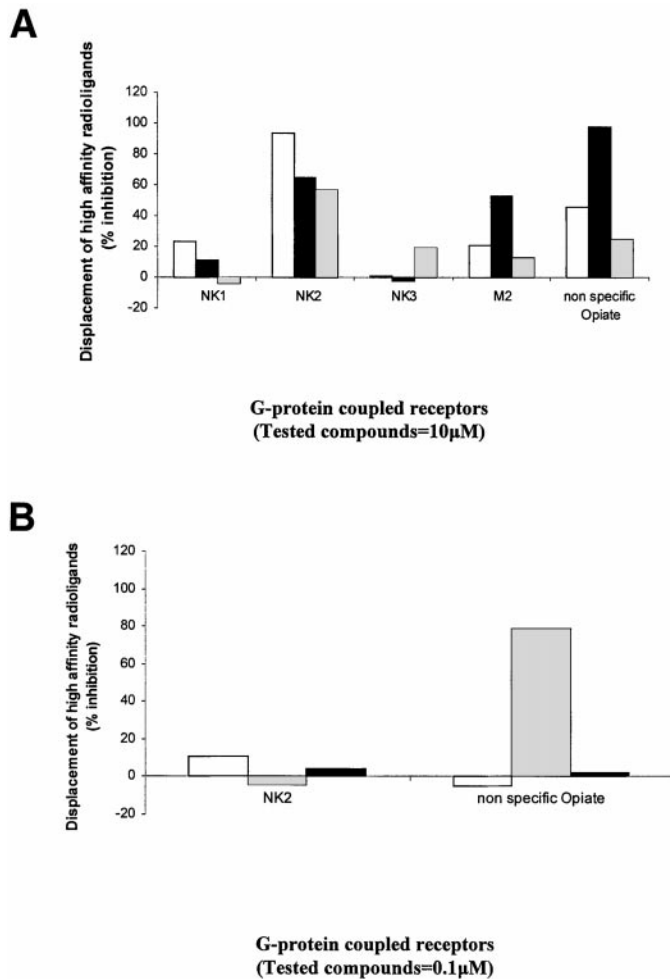


FIG. 7. NovaScreen (Hanover, MD) performed the assessments for nonspecific binding to various G protein-coupled receptor families. Radioligand displacement studies were performed with neurokinin receptors: NK1, rat submaxillary gland; NK2, human recombinant; and NK3, rat cortex with [³H]substance P ($K_i = 6.76 \times 10^{-9}$ M), [¹²⁵I]NK-A ($K_i = 1.27 \times 10^{-9}$ M), and [¹²⁵I]eleidoisin ($K_i = 3.21 \times 10^{-9}$ M) as the specific radioligands, respectively. The opiate nonselective receptor with membranes isolated from the rat forebrain using [³H]naloxone ($K_i = 1.45 \times 10^{-9}$ M) and the muscarinic M2 receptor with membranes isolated from the rat heart using [³H]AF-DX 384 ($K_i = 1.65 \times 10^{-8}$ M) were used as radioligands. The unlabeled compounds SRIF (□), octreotide (▣), and PTR-3173 (■) were tested in primary assays with final concentrations of 10^{-5} M (A) and 10^{-7} M (B) for their competition potency with the specific radioligand binding.

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-

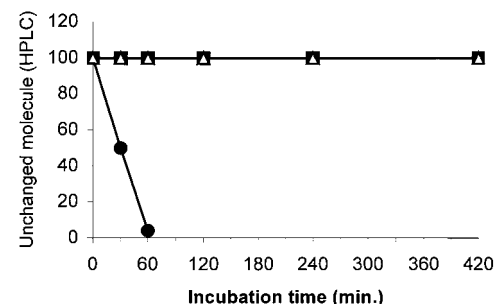


FIG. 8. The metabolic stability of SRIF-14 (●), octreotide (■), and PTR-3173 (▲) was tested against degradation by enzymes in human serum. The tested compounds were incubated in human serum at 37°C, samples were withdrawn at different time intervals, and the percentage of unchanged molecule was measured by HPLC.

FIG. 9. The pharmacokinetic profiles of PTR-3173. Plasma concentrations of PTR-3173 were plotted against a time scale after iv bolus (■) and sc (○) administrations to conscious Wistar rats as described in *Materials and Methods*.

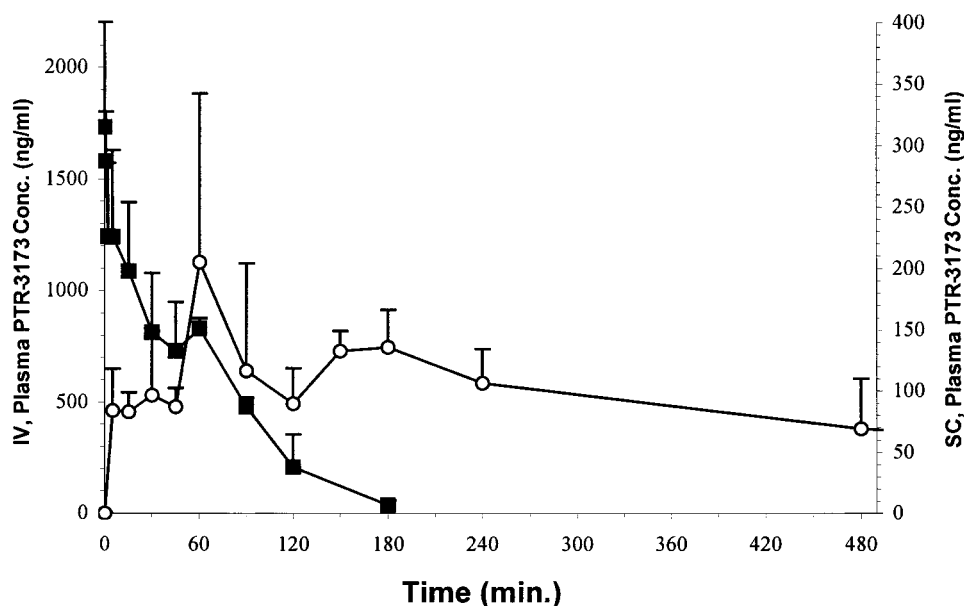


TABLE 1. Pharmacokinetic constants of PTR-3173 and octreotide in rats

Route	Drug	F (%)	V _{ss} (ml/kg)	t _{1/2} (min)	% Excreted in urine	CL (ml/min·kg)
iv	PTR-3173		653	31	10.3	13.0
	Octreotide		602	49	21.3	17.6
sc	PTR-3173	99.6		170	15.9	13.3
	Octreotide	103		40	23.0	17.1

F, Bioavailability; V_{ss}, volume of distribution; T_{1/2}, circulating half-life; CL, clearance. The pharmacokinetic values of octreotide presented here adapted from Ref. 42.

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 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 $\mu\text{g}/\text{kg}$, which is above the reported ED₅₀ of octreotide for inhibition of GH (0.1 $\mu\text{g}/\text{kg}$), glucagon (0.65 $\mu\text{g}/\text{kg}$), or insulin (26 $\mu\text{g}/\text{kg}$) 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 $\mu\text{g}/\text{kg}$ 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 $\mu\text{g}/\text{kg}$ similar to octreotide, glucagon release was inhibited only at 100 $\mu\text{g}/\text{kg}$. 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 $\mu\text{g}/\text{kg}$); 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,

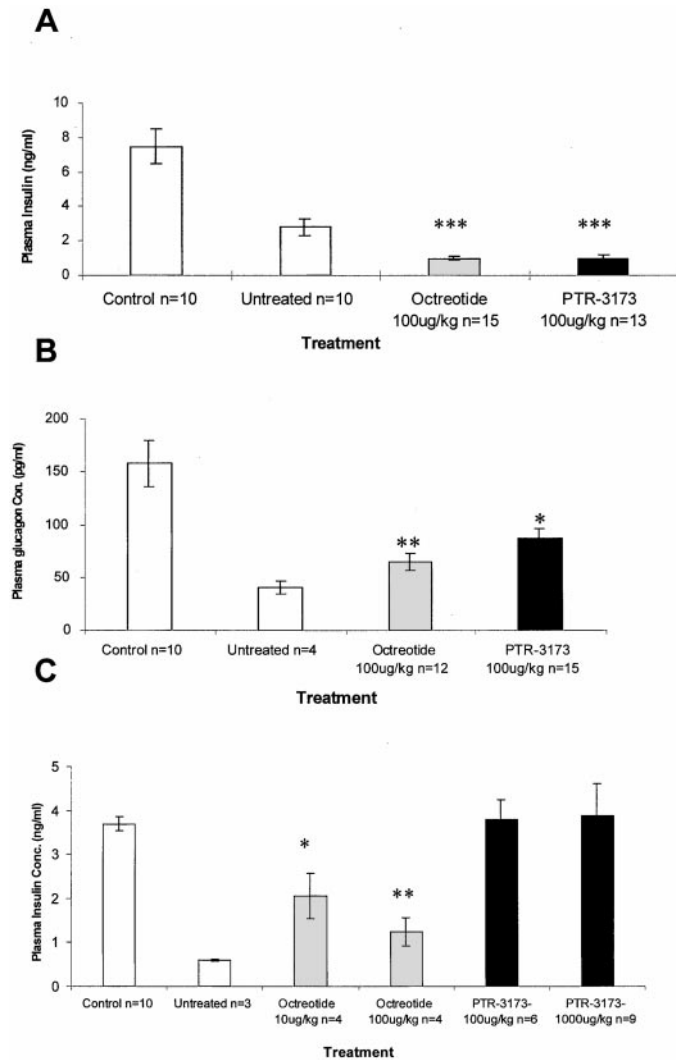


FIG. 10. The pharmacodynamics of PTR-3173 compared with octreotide were evaluated by hormone release assays performed with Wistar rats under Nembutal anesthesia. Plasma GH and glucagon levels were measured in the same blood samples, which were collected from fasted animals at 5 min after iv administration of L-arginine. Plasma insulin levels were measured by a separate experiment; blood was collected 5 min after D-glucose was administered iv. Octreotide or PTR-3173 was administered sc at 15 min before blood collection with an equal dose of 100 μ g/kg. A, Plasma GH levels at 5 min after L-arginine administration to control animals (\square) compared with the nonstimulated animals (untreated) and those given PTR-3173 (\blacksquare) and octreotide (\blacksquare). 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 ED₅₀ of octreotide of insulin release was 26 μ g/kg (31), which was confirmed by our study. PTR-3173, at 100 or 1000 μ g/kg, did not affect insulin release. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (vs. control).

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.

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 vitro* 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 sst2 receptor subtype mediates SRIF inhibition of GH and glucagon (25, 43, 44) secretion. Rossowski *et al.* (45) and Strowski *et al.* (44) demonstrated insulin secretion inhibition in rodents by sst5-selective SRIF analogs. Other studies performed with human pancreatic islets demonstrate that sst2 is also related to insulin secretion inhibition by SRIF (17). As the affinities of human and rat sst5 receptors toward SRIF analogs 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 sst5. 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 sst2, sst5, or a combination of these receptors plays the principal role in insulin secretion in the rat has yet to be determined.

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

Effect	GH ED ₅₀ (μg/kg)	Glucagon ED ₅₀ (μg/kg)	Insulin ED ₅₀ (μg/kg)	Potency ratio GH/insulin	Potency ratio GH/glucagon
Octreotide	0.08	0.65	26	309	8
PTR-3173	0.1	100	>1,000	>10,000	1,000

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.

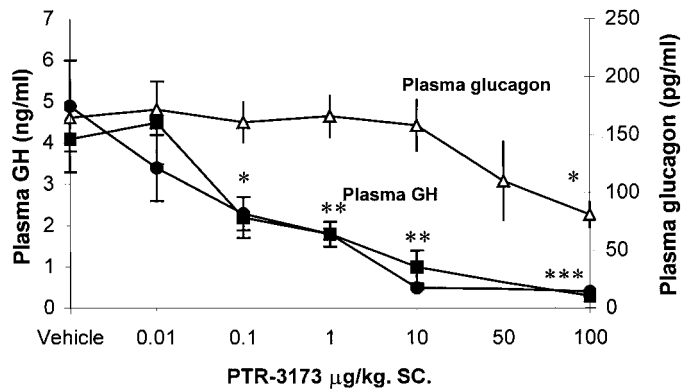


FIG. 11. Dose-response relationships of PTR-3173 on L-arginine-induced GH and glucagon release in Wistar rats. ED₅₀ values were measured using the experimental protocols of hormone release described in *Materials and Methods*. PTR-3173 reduced plasma GH levels with ED₅₀ of 80–100 ng/kg after 15 (●) and 30 (■) min of drug administration. The dose-response studies with glucagon (Δ) 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 sst2 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 counterregulatory 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 dependent (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.

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