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A new peptidic somatostatin agonist with high affinity to all five somatostatin receptors

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Abstract

All commercially available somatostatin analogs for clinical use have a preference for some but not all somatostatin receptor subtypes. We describe here the synthesis and evaluation in binding and cAMP assays with cell lines stably transfected with sst_1–sst_5 of a new type of nonapeptide somatostatin analog with a reduced-sized and stabilized structure, Tyr^0–(cyclo-D-Dab–Arg–Phe–Phe–D-Trp–Lys–Thr–Phe) (KE108). All five somatostatin receptors subtypes have an extremely high affinity for KE108, equivalent to SS-28 at sst_1 and two to four times higher than SS-28 at sst_2, sst_3, sst_4 and sst_5. Moreover, the compound has agonistic properties at all five subtypes, since it is able to inhibit the forskolin-stimulated cAMP production in sst_1–sst_5 cells. It is stable for several hours in human serum. This analog may therefore represent a considerable improvement over commercially available somatostatin analogs as it will target all somatostatin receptor subtypes, a particular advantage for cancer-related applications, as human cancers can express concomitantly several somatostatin receptor subtypes.

Keywords: Somatostatin receptor subtype; Cancer; Octreotide; Peptide; Peptide receptor targeting

1. Introduction

In the past 20 years, several somatostatin analogs have been developed for clinical use. Unlabeled octreotide and lanreotide are being used for the symptomatic treatment of neuroendocrine tumors (Lamberts et al., 1996; Oberg, 1998). More recently, ^{111}In- and ^{90}Y-labeled, chelator-bound octreotide and lanreotide have been used for the diagnosis and radiotherapy of somatostatin receptor-expressing tumors (Krenning et al., 1995, 1996; Otte et al., 1998; Paganelli et al., 2001; Traub et al., 2001). There are, however, at least five different somatostatin receptor subtypes (Hoyer et al., 1995), which may be expressed concomitantly and in various combinations in normal or pathological somatostatin targets (Patel, 1999; Reubi et al., 2001; Schaer et al., 1997). It is therefore of prime interest to either develop analogs selective for one particular subtype (Rohrer et al., 1998), for use in restricted indications, or develop analogs that bind equally well to all five subtypes (pan-somatostatin), for broader indications and, perhaps, to take advantage of multiple somatostatin receptor subtype interactions, such as homo- and heterodimerisation (Pfeiffer et al., 2001; Rocheville et al., 2000). Unfortunately, successful somatostatin analogs commercially available for clinical use have a preference for some of the somatostatin receptor subtypes (sst_2, sst_3, sst_5) but not for others (sst_1, sst_4) (Hoyer et al., 1995). The search for somatostatin analogs with a high affinity binding profile to all five subtypes was only started a few years ago. Some somatostatin analogs have been claimed to correspond almost to a pan-somatostatin, such as ^{111}In-1,4,7,10-tetraazadodecane 1,4,7,10-tetraacetic acid (DOTA)-lanreotide (Mauritius) (Traub et al., 2001) or depreotide (P829) (Blum et al., 2000). Binding studies with sst_1–sst_5-transfected cells have, however, revealed that these analogs still retained a low affinity for sst_1 and sst_4 subtypes (Reubi et al., 2000a) as is also the case for octreotide. In fact, when used in

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the clinic, compounds like ^{111}In-DOTA-lanreotide or ^{99}Tc-P829 behave only marginally differently or are considerably less adequate than the corresponding octreotide analogs (Lebtahi et al., 2002; Virgolini et al., 2001). A further search for adequate pan-somatostatins was therefore indicated. In the present study, we describe a novel somatostatin analog that binds with high affinity to all five somatostatin receptor subtypes and has agonistic properties to all five subtypes.

2. Materials and methods

2.1. Reagents

All chemicals were obtained from commercial sources and used without further purification. TCP (tritylchloride-crosslinked polystyrene) was purchased from PepChem (Tübingen, Germany). Fmoc (9-fluorenylmethoxycarbonyl)-amino acids were purchased from Nova Biochem (Läufelfingen, Switzerland) or from Bachem (Bubendorf, Switzerland).

2.2. Chemical syntheses

2.2.1. Synthesis of benzyloxycarbonyl-D-diaminobutyric acid (Fmoc)-OH

Benzyloxycarbonyl-D-diaminobutyric acid-OH (3 g) was dissolved in acetone/water 1:1 (16 ml each), and sodium carbonate (1.3 g) was added to a final pH of 9–10. Fmoc-*N*-hydroxysuccinimide ester (4 g) was added and the reaction mixture was stirred for 18 h at RT and cooled to 5 °C; ethyl acetate (35 ml) was added and acidified with 6N HCl (pH 3). The organic phase was washed four times with water, dried over sodium sulfate and concentrated. The product was recrystallized from ethyl acetate/petrolether. Yield is 5.3 g (94%).

2.2.2. Synthesis of KE108

The solid phase peptide synthesis was carried out on a semiautomatic peptide synthesizer. The linear peptide intermediate was assembled on a trifluoro-acetic acid (TFA) resin starting with Fmoc–Phe-OH. Fmoc was used as a transient amino protecting group. In addition, TFA-labile side chain-protecting groups were utilized, such as 2,2,4,6,7-pentamethyl-dihydro-benzofuran-5-sulfonyl for Arg, *tert*-butoxycarbonyl for Trp and Lys, *tert*-butyl for Thr. Coupling reactions were done with in situ prepared hydroxybenzotriazole esters using diisopropylcarbodiimide. The assembled peptides were cleaved mildly from the resin, using 20% acetic acid in dichloromethane, and co-evaporated twice with toluene (50 ml) to remove acetic acid. Subsequently, cyclization was performed via carboxamide formation, using 10 equivalents of dicyclohexylcarbodiimide/hydroxybenzotriazole in dimethylformamide (DMF) at high dilution. The crude product was dissolved in a 5% aqueous oxalic acid solution, extracted three times with ethyl acetate and the organic layer was evaporated to dryness. The benzyloxycarbonyl-protecting group was selectively removed by catalytic hydrogenolysis, using palladium/charcoal as catalyst in methanol. The products were filtered and purified with a SepPak cartridge C_{18} (Macherey-Nagel, Düren, Germany), using water and methanol as eluents. The resulting free amino acid group serves as an attaching point for the coupling of Boc–Tyr(tBu)-OH. Deprotection was performed with a solution of trifluoroacetic acid/phenol/thioanisol/water 85:5:5:5 for 2–5 h (Fig. 1A). The final product was precipitated from isopropyl ether/petrolether 1:1 and purified by C_{18} reverse-phase chromatography (Metrohm LC CaDi

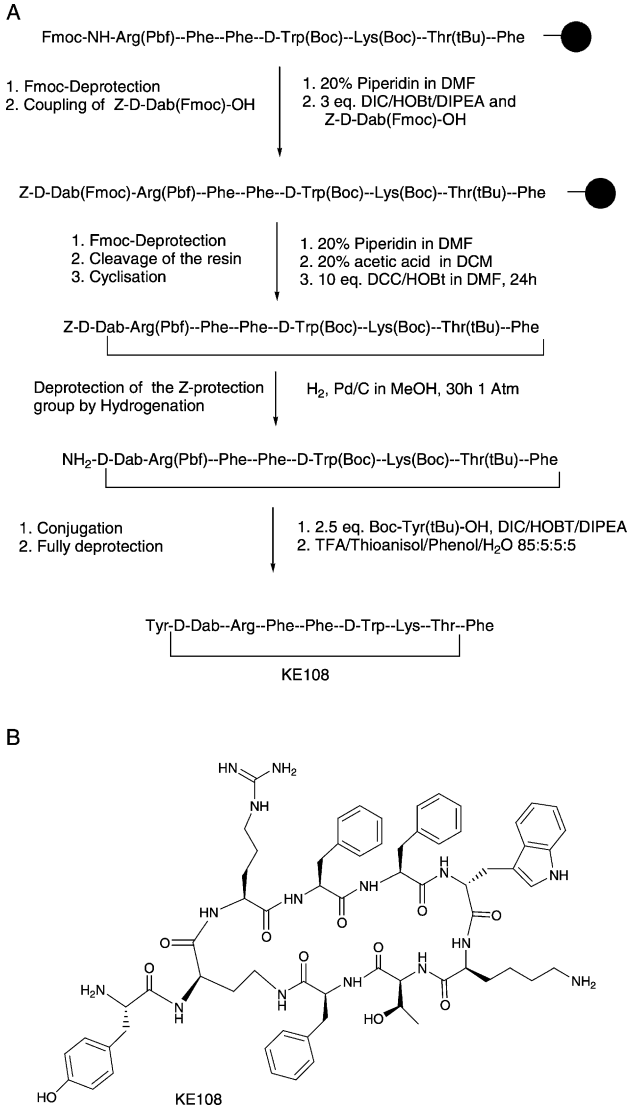

Fig. 1. (A) Synthesis of KE108. Z, benzyloxycarbonyl; Fmoc, fluorenylmethoxycarbonyl; Dab, diaminobutyric acid; DIC, diisopropylcarbodiimide; Pbf, 2,2,4,6,7-pentamethyl-dihydro-benzofuran-5-sulfonyl; Boc, *tert*-butoxycarbonyl; tBu, *tert*-butyl; HOBt, *N*-hydroxybenzotriazole; DIPEA, diisopropylethylamine; OSu, *N*-hydroxysuccinimide ester. (B) Structure of KE108.

22-14, column: Macherey-Nagel) with a purity >93% and characterized by electro-spray ionization mass spectroscopy (ESI-MS) and reverse-phase high-pressure liquid chromatography (RP-HPLC) (Fig. 1B).

ESI-MS(+):1278 (12, $[M+H]^+$); 639 (100, $[M+2H]^{2+}$).

ESI-MS(−): 1504 (30, $[M+2TFA]^-$); 1389 (20, $[M+TFA]^{2-}$).

K119 was synthesized accordingly, using Boc−3-I-Tyr(tBu)-OH).

ESI-MS(+): 702 (100, $[M+2H]^{2+}$).

ESI-MS(−): 1630 (100, $[M+2TFA]^-$); 1515 (85, $[M+TFA]^{2-}$).

2.3. Binding affinity determination to sst_1–sst_5

Cells stably expressing human sst_1, sst_2, sst_3, sst_4 and sst_5 (CHO-K1 for sst_1 and sst_5, CCL39 for sst_2, sst_3 and sst_4) were grown as described previously (Reubi et al., 2000a). All culture reagents were supplied by Gibco and Life Technologies (Grand Island, NY). Cell membrane pellets were prepared and receptor autoradiography was performed on pellet sections (mounted on microscope slides), as described in detail previously (Reubi et al., 2000a). Complete displacement experiments were performed on successive pellet sections with the universal somatostatin radioligand ^{125}I-[Leu^8, D-Trp^{22}, Tyr^{25}]-somatostatin-28 (2000 Ci/mmol; Anawa, Wangen, Switzerland), using 30,000 cpm/100 μl and increasing concentrations of the unlabeled KE108 ranging from 0.1 to 1000 nM. The unlabeled, universal somatostatin-28 was run as control in parallel, using the same increasing concentrations. After quantification of the data with a computer-assisted image processing system (Reubi et al., 1990, 2000a), displacement curves were obtained, from which the IC_{50} values were calculated (Reubi et al., 2000a). Tissue standards (Autoradiographic [^{125}I] microscales; Amersham) that contain known amounts of isotope, cross-calibrated to tissue-equivalent ligand concentrations, were used for quantification (Reubi, 1995). Advantages of the present method using receptor autoradiography with sectioned cell pellets compared with binding on cell homogenates are, in addition to an economic use of cells and a great flexibility, its greater interassay reliability and reproducibility, because the same embedded pellet can be used for successive experiments. A minor disadvantage is that IC_{50} values are somewhat higher than in the homogenate binding assay (Reubi et al., 2000a).

2.4. Adenylate cyclase activity

The effect of KE108 on forskolin-stimulated cAMP formation was performed on sst_1–sst_5 transfected cells, as described previously (Reubi et al., 2000b). sst_1–sst_5-expressing cells were subcultured in 96-well culture plates at 2×10^4 cells per well and grown for 24 h. Culture medium was removed from the wells and fresh medium (100 μl) containing 0.5 mM 3-isobutyl-I-methylxanthine (IBMX) was added to each well. Cells were incubated for 30 min at 37 °C. Medium was then removed and replaced with fresh medium containing 0.5 mM IBMX, with or without 10 μM forskolin and various concentrations of peptides. Cells were incubated for 30 min at 37 °C. After removal of the medium, cells were lysed and cAMP accumulation was determined using a commercially available cAMP scintillation proximity assay (SPA) system (RPA 538), according to the instructions of the manufacturer (Amersham, Aylesbury, UK). cAMP data were expressed as percentages of stimulation over the nonstimulated level. Values of EC_{50} (the agonist concentration causing 50% of its maximal effect) were derived from the concentration–response curves (Reubi et al., 2000b).

3. Results

The structure of KE108 is shown in Fig. 1B. It is a nonapeptide with a Tyr^0 attached to the D-Dab^1 that is cyclized with Phe^8. It contains the Phe–D-Trp–Lys–Thr motif, crucial for high-affinity somatostatin receptor binding. The peptide was tested in displacement experiments for its ability to bind to the five human cloned somatostatin receptors. The respective IC_{50} were compared with that of SS-28 performed in the same experiment as control. Table 1 shows that the binding affinity of KE108 is equal to or higher than that of SS-28 for the five subtypes. KE108's binding affinity is equivalent to SS-28 at sst_1 and two to four times higher than SS-28 at sst_2, sst_3, sst_4 and sst_5. The corresponding 3-I-Tyr^0 analog of KE108 (KE119) has comparable, though slightly lower binding affinity values at sst_1–sst_5 (Table 1). Fig. 2 shows that KE108 behaves similarly to SS-28 in inhibiting forskolin-stimulated cAMP accumulation. Not only does it inhibit stimulated cAMP to the same levels than SS-28, but it also does it at similar concentrations as illustrated in Fig. 2. EC_{50} for KE108 for

Table 1
Binding affinity of KE108 and K119 to all sst subtypes, compared to SS-28 (expressed as IC_{50}, nM; mean ± S.E.M.); number of experiments in parenthesis

	sst_1	sst_2	sst_3	sst_4	sst_5
SS-28	2.7 ± 0.3 (12)	2.3 ± 0.2 (12)	3.4 ± 0.3 (12)	2.7 ± 0.3 (12)	2.5 ± 0.3 (12)
KE108	2.6 ± 0.4 (7)	0.9 ± 0.1 (7)	1.5 ± 0.2 (7)	1.6 ± 0.1 (7)	0.65 ± 0.1 (7)
KE119	6.9 ± 1.1 (3)	1.1 ± 0.3 (3)	2.5 ± 0.5 (3)	3.8 ± 0.4 (3)	2.6 ± 0.6 (3)

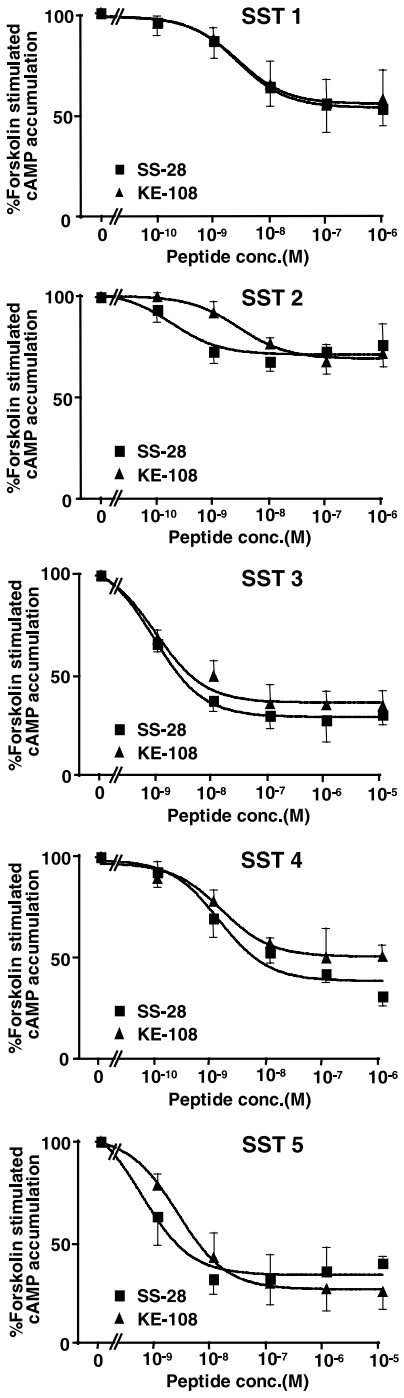

Fig. 2. Effect of KE108 and SS-28 on forskolin-stimulated cAMP production in sst_1–sst_5 cells. Concentration–response curves were obtained by increasing concentrations of SS28 (■) and KE108 (▲). Data from three separate experiments in duplicate (mean ± S.E.M.) are expressed as a percentage of the 10 μM forskolin response. KE108 has similar agonistic properties than SS-28 on each of the sst subtypes.

the inhibition of forskolin-stimulated cAMP were, respectively, 3.44 ± 2.1 nM at sst_1, 3.53 ± 0.46 nM at sst_2, 1.64 ± 0.09 nM at sst_3, 1.61 ± 0.18 nM at sst_4 and 3.07 ± 0.92 nM at sst_5 (mean ± S.E.M.). The stability of the corresponding peptide containing a chelator was tested by incubating for 24 h in human serum. HPLC detection revealed that the major peak was intact peptide, indicating that the compound was stable for several hours in human serum in vitro. We can conclude that KE108 is a potent and stable somatostatin receptor agonist at all five receptor subtypes.

4. Discussion

The present study reports on an extremely potent somatostatin analog that acts as an agonist at all five somatostatin receptor subtypes. Remarkably, compared to other reduced-sized and metabolically stabilized somatostatin analogs such as octreotide, lanreotide, vapreotide, or even the new SOM230 (Bruns et al., 2002), KE108 has not only a very high affinity for sst_1 and sst_4 but also a remarkably improved affinity for sst_3 and sst_5. The structural requirements for these massive improvements are not clear to date.

Such a compound may be used in different situations: first, due to its very strong effect on a second messenger system related to all five somatostatin receptor subtypes, such as cAMP production, KE108 is likely to have an efficient functional role and may be a useful drug as unlabeled compound, to be developed for long-term therapy in somatostatin receptor-positive tumors as an alternative to the commercially available octreotide or lanreotide. Preferential indications are tumors that express concomitantly several different somatostatin receptor subtypes. However, it may as well be worth using it in tumors with predominant expression of sst_1 such as prostate carcinomas or sarcomas (Reubi et al., 2001), or of sst_3 expression such as inactive pituitary adenomas (Reubi et al., 2001; Sharma et al., 1996). Clinical trials will have to demonstrate whether KE108 is able to inhibit tumor progression to a larger extent than previously shown by octreotide or lanreotide and whether it is able to adequately inhibit hormone release, as these two commercially available analogs successfully do. Moreover, a pan-somatostatin may also be effective in non-oncological indications characterized by disturbances of the physiological somatostatin system. These may include immune diseases or gastrointestinal disturbances, namely, systems with expression of selective somatostatin receptor subtypes such as sst_1 and sst_3 (Corleto et al., 1999; Murthy et al., 1996; Reubi et al., 2000b, 2001). More precise formulation of clinical non-cancer indications will rely on additional basic information on somatostatin function in healthy tissues. Second, because of its Tyr in position zero, the present pan-somatostatin may easily be labeled with radioactive iodine (the corresponding KE108 analog labeled with cold iodine has also pan-somatostatin properties; see KE 119, Table 1) and could be used as radiotracer in vitro and in vivo in order to identify the tissues expressing all five subtypes. Since it was shown that ^{111}In- and ^{90}Y-chelator-linked octreotide radiotracers were far superior in terms of their biodistribution in vivo in humans compared to their iodi-

nated counterparts (Krenning et al., 1995), the development of chelator-linked KE108 will be given first priority (Heppeler et al., 1999). However, the in vivo characterization of KE108, such as its in vivo stability, metabolism, biodistribution and toxicology, will represent further necessary steps before clinical applications will be started.

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