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Cellular effects of AP102, a somatostatin analog with balanced affinities for the hSSTR2 and hSSTR5 receptors

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ABSTRACT

Background: Somatostatin analogs (SSAs) are first-line medical therapy for the treatment of acromegaly and neuroendocrine tumors that express somatostatin receptors (SSTR). Somatostatin suppresses secretion of a large number of hormones through the stimulation of the five SSTR. However, unbalanced inhibition of secretion as observed with the highly potent SSAs pasireotide causes hyperglycaemia mainly by inhibiting insulin secretion. In contrast, AP102 a new SSAs has neutral effect on blood glucose while suppressing GH secretion. Our objective was to establish the cellular effects of AP102 on SSTR2 and SSTR5 that may explain the differences observed between AP102 and other SSAs.

Methods: We compared the binding and agonist activity of AP102 with somatostatin-14, octreotide and pasireotide in HEK293 cells transfected with human SSTR2 and SSTR5 receptors. SSAs signal transduction effects (cAMP concentrations) were measured in forskolin-treated cells in the presence of SSAs. Proliferation and apoptotic effects were determined and binding assays were performed using ^{125}I- somatostatin-14.

Results: AP102 has comparable affinity and agonist effect to octreotide at SSTR2 (IC50's of 112 pM and 244 pM, respectively; EC50's of 230 pM and 210 pM, respectively) in contrast to pasireotide that exhibits a 12–27 fold higher IC50 (3110 pM) and about 5-fold higher EC50 (1097 pM). At SSTR5, AP102 has much higher affinity and stimulating effect than octreotide (IC50's of 773 pM and 16,737 pM, respectively; EC50's of 8526 pM and 26,800 pM), and an intermediate affinity and agonist effect between octreotide and pasireotide. AP102, octreotide and pasireotide have variable anti-proliferative effects on HEK cells transfected with SSTR2 and SSTR5.

Conclusion: AP102 is a new SSA that better reduces signaling at SSTR2 than SSTR5 and prevents cell proliferation at both receptors. The euglycaemic effect of AP102 observed in preclinical studies may be related to this intermediate agonistic potency between pasireotide and octreotide at SSTR2 and SSTR5.

1. Introduction

Somatostatin analogs (SSAs), also known as somatostatin-receptor ligands (SRL), are used in the treatment of diseases like acromegaly, caused mostly to chronic growth hormone (GH) hypersecretion from a pituitary tumor (Katznelson et al., 2014; Öberg and Lamberts, 2016). They are also used in the management of neuroendocrine tumors (NET) that secrete large amounts of bioactive neurotransmitters (*e.g.* catecholamines or serotonin) (Eisenhofer et al., 2012; Franscini et al., 2015; Modlin et al., 2016). Pituitary tumors and NET express somatostatin receptors (SSTR) and activation of these receptors can have anti-secretory and anti-proliferative effects (Körner, 2016; Csaba and Dournaud, 2001). These properties led to the development of octreotide and lanreotide that are SSTR2 specific, while pasireotide is an agonist with activity at multiple SSTRs, predominantly SSTR5, but with a lower SSTR2 affinity than first generation compounds (Öberg and Lamberts, 2016; Colao et al., 2016). SSTR2-specific compounds have incomplete efficacy in the clinical setting, presumably due to variable expression SSTR2 by tumors and their low affinity for other biologically important receptors like SSTR5 (Paragliola et al., 2017). Pasireotide has greater efficacy than octreotide in acromegaly, but this is associated with the development of hyperglycemia and diabetes *via* its strong SSTR5

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activity that inhibits insulin secretion (McKeage, 2015; Silverstein, 2016).

AP102 is a disulfide-bridged iodinated octapeptide that contains synthetic amino acids selected from a library of 10 SSAs (identified as peptide #9 in reference (Moore et al., 2005)). Receptor binding studies conducted using membranes from rat colon carcinoma cells (CC531 cells) (for hSSTR2) and Chinese hamster ovary (CHO)-K1 cells (for hSSTRs 1, 3, and 5), stably expressing individual human somatostatin receptor subtypes established sub-nanomolar affinity for both the SSTR2 and SSTR5 receptors (Moore et al., 2005). Moreover, AP102 exhibited virtually identical inhibitory effects on GH and prolactin release as native somatostatin-14 (SS14) in cultured pituitary cells obtained from female Wistar rats (Moore et al., 2005). When AP102 was administered to healthy male rats it acutely reduced growth hormone secretion, but did not result in hyperglycemia when given acutely or chronically (Tarasco et al., 2017). Our results demonstrate the potential for AP102 to fill the gap left by other SSAs in treating conditions associated with hormonal hypersecretion. We postulated that a different affinity and cellular effect at the SSTR2 and SSTR5 receptors with AP102 as compared to pasireotide could explain the absence of diabetogenic effects, while permitting relevant effects on hormonal suppression.

The aim of this study was to compare AP102 with octreotide and pasireotide regarding cellular effects, including cell signal transduction, cell proliferation and toxicity using stably-transfected HEK 293 cells expressing hSSTR2 and hSSTR5.

2. Materials and methods

2.1. Compounds

AP102 was synthesized as described (Moore et al., 2005). Somatostatin-14 (also known as somatotropin release inhibiting factor 14 or SRIF-14) was purchased from Polypeptide laboratories (Strasbourg, France) and octreotide was purchased from Sigma (Sigma-Aldrich, Buchs, Switzerland). Pasireotide (also known as SOM230) was obtained from a vial of Signifor® (Novartis, Basel).

2.2. HEK-293 cell lines expressing hSSTR2 and hSSTR5

Plasmids encoding the human somatostatin receptors 2 and 5 transgenes were obtained from Missouri S&T cDNA resource center: http://www.cdna.org hSSTR2: ref. number hSSTR20TN00; hSSTR5: ref. number hSSTR50TN00. Both transgenes contained an N-terminal 3xHA-tag and were cloned into a pcDNA3.1 + vector. Transfections were performed with lipofectamine 2000 (Invitrogen, Luzern, Switzerland) according to the manufacturer's protocol. After transfection, cells were cultivated in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate (Life Technology, Zug, Switzerland) and incubated in a humidified 5% CO_2 incubator at 37 °C. Positive clones were selected with 500 µg/ml of G418 (Gentaur, Zürich, Switzerland) and tested for the presence of SSTR transgenes as follows: cells were lyzed in a buffer containing PBS with 0.5% Triton-X100 and protease inhibitor with EDTA (Roche, Basel, Switzerland). Cell lyzates were fractionated with SDS-PAGE under reducing conditions using precast gels (Bio-Rad, Reinach, Switzerland), proteins were electroblotted onto nitrocellulose membrane (Bio-Rad) and probed with anti-SSTR antibodies. SSTR proteins were immuno-detected using rabbit anti-SSTR2 (AB 134152, Abcam, Cambridge, United Kingdom) and anti-SSTR5 (AB 109495, Abcam) monoclonal antibodies. A monoclonal antibody against β-actin was obtained from Sigma (A-5441). Secondary HRP-conjugated antibodies (#170-6515) were purchased from Bio-Rad (Reinach, Switzerland). Immunoreactive bands were revealed by a chemiluminescence assay (Western lightning plus-ECL, PerkinElmer, Schwerzenbach, Switzerland) and the signal was processed by a digital imaging analyzer (ImageQuant LAS-4000, General Electric, Glattbrugg, Switzerland). Expression of hSSTR2 and hSSTR5 was successfully detected in transfected cells (Fig. S1).

2.3. Binding assays

Cells were harvested at 80% confluency in a 145 cm^2 petri dishes, washed with 20 ml cold PBS, scraped with 3 ml of cold harvesting buffer (HB), and 1.25 ml HB was used to rinse the petri dish. The HB composition was as follows: 50 mM hepes pH 7.5, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.50% BSA (Sigma-Aldrich), 150 mM NaCl, 2.5 µgr/ml Aprotinin (Applichem, Darmstadt, Germany) and 250 µg/ml Bacitracin (Sigma-Aldrich). Cells were centrifuged at 1000 rpm for 2 min after which the pellet was re-suspended (1 ml/dish with HB and 5% DMSO) and samples were aliquoted at 150 µl and stored at −80 °C. Protein concentration was quantified at 450 µg/ml for hSSTR2 and 700 µg/ml for hSSTR5 with a BCA test (Thermofisher, Basel, Switzerland).

SS14 and tested SSAs (AP102, octreotide and pasireotide) were diluted in binding buffer (BB) containing 5 mM Hepes pH 7.5, 5 mM MgCl2, 2 mM CaCl2, 0.10% BSA, 2.5 µgr/ml aprotinin and distributed (150 µl) in duplicate Eppendorf tubes. To this was added 15,000 cpm of ^{125}I-SS14 (100 µl) (10 µCi, 2200 ci/mmol, PerkinElmer) diluted in BB and finally the cell membrane preparations were added (diluted in 50 µl of BB and corresponding to 4.5 µg and 7 µg of protein for hSSTR2 and hSSTR5, respectively) to reach a total incubation volume of 300 µl. These underwent rolling incubation for 45 min at room temperature and cell membranes were pelleted after 2 min of centrifugation at 14000 rpm in a microfuge. The supernatant was discarded and cell membranes were shaken and washed with 300 µl BB before another round of centrifugation and supernatant removal. Membrane pellets were cut from the bottom of the Eppendorf tubes and were placed in a tube for counting in a gamma radioactivity counter (GammaCounter Wizard 1470-020, PerkinElmer) for 1 min.

Non-specific binding was determined in presence of 1 µM of SS14 and specific binding corresponding to the difference between total binding (cpm measured in tube without competitor) and non-specific binding.

2.4. cAMP accumulation experiments

HEK-hSSTR2 or HEK-hSSTR5 cells were seeded in 24-well plates at 375,000 cells per well and grown in DMEM supplemented with 10% fetal bovine serum for 48 h in a humidified 5% CO_2 incubator at 37 °C. Culture medium was then removed, and 200 µl of fresh serum-free DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine (buffer A; Sigma-Aldrich) was added to each well. After five minutes' incubation at 37 °C in 5% CO2, 100 µl of buffer A containing varying concentrations of SSAs was added. In order to stimulate adenylate cyclase, 100 µl of fresh buffer A containing 40 µM forskolin (Sigma-Aldrich) was added and the cells were further incubated for 20 min at 37 °C. The cell medium was removed and 200 µl of 0.1 M HCl were added to each well to ensure stabilization of cAMP and allow its extraction. Cells were dissociated by pipetting up and down until suspension was homogeneous. The lysate was transferred to a centrifuge tube and washed thoroughly with 100 µl of 0.1 M HCl to recover remaining traces of sample; thereafter the extract was centrifuged at 14000 rpm for 10 min (200 µl + 100 µl). The supernatants were dried by lyophilisation and reconstituted in 100 µl of HCl 0.1 M before assays. cAMP accumulation was measured using the cAMP direct immunoassays kit (colorimetric, K371; BioVision) according to the manufacturer's protocol. The maximum of forskolin-stimulated cAMP formation was defined in the presence of 10 µM forskolin and the maximum inhibition was obtained using 100 nM of SS14. EC50's were calculated using GraphPad Prism 6 software based on 3–4 separate curves using five concentrations of ligands in duplicate (*i.e.*, two separate wells per concentration) (Fig. S2).