European Journal of Pharmacology 644 (2010) 257–262

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Endocrine Pharmacology

Absence of somatostatin SST_2 receptor internalization *in vivo* after intravenous SOM230 application in the AR42J animal tumor model

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ARTICLE INFO

Article history:
Received 16 March 2010
Received in revised form 1 July 2010
Accepted 9 July 2010
Available online 16 July 2010

Keywords:
Somatostatin receptor
Internalization
Tumor targeting
SOM230
Octreotide
Functional selectivity
Desensitization

ABSTRACT

Among clinically relevant somatostatin functions, agonist-induced somatostatin receptor subtype 2 (sst_2) internalization is a potent mechanism for tumor targeting with sst_2 affine radioligands such as octreotide. Since, as opposed to octreotide, the second generation multi-somatostatin analog SOM230 (pasireotide) exhibits strong functional selectivity, it appeared of interest to evaluate its ability to affect sst_2 internalization *in vivo*. Rats bearing AR42J tumors endogenously expressing somatostatin sst_2 receptors were injected intravenously with SOM230 or with the [Tyr^3, Thr^8]-octreotide (TATE) analog; they were euthanized at various time points; tumors and pancreas were analyzed by immunohistochemistry for the cellular localization of somatostatin sst_2 receptors. SOM230-induced sst_2 internalization was also evaluated *in vitro* by immunofluorescence microscopy in AR42J cells. At difference to the efficient *in vivo* sst_2 internalization triggered by intravenous [Tyr^3, Thr^8]-octreotide, intravenous SOM230 did not elicit sst_2 internalization: immunohistochemically stained sst_2 in AR42J tumor cells and pancreatic cells were detectable at the cell surface at 2.5 min, 10 min, 1 h, 6 h, or 24 h after SOM230 injection while sst_2 were found intracellularly after [Tyr^3, Thr^8]-octreotide injection. The inability of stimulating sst_2 internalization by SOM230 was confirmed *in vitro* in AR42J cells by immunofluorescence microscopy. Furthermore, SOM230 was unable to antagonize agonist-induced sst_2 internalization, neither *in vivo*, nor *in vitro*. Therefore, SOM230 does not induce sst_2 internalization *in vivo* or *in vitro* in AR42J cells and pancreas, at difference to octreotide derivatives with comparable sst_2 binding affinities. These characteristics may point towards different tumor targeting but also to different desensitization properties of clinically applied SOM230.

1. Introduction

Stable and potent somatostatin analogs such as octreotide and lanreotide are widely used for the successful symptomatic treatment of neuroendocrine tumors (Eriksson and Oberg, 1999; Freda, 2002; Reubi, 2003). More recently, second generation multi-somatostatin analogs, such as KE108 or SOM230 (pasireotide), have been developed having a high affinity for several or all five somatostatin receptor subtypes (Lewis et al., 2003; Reubi et al., 2002; Schmid, 2008). Indeed, KE108 displays high affinity to all 5 somatostatin receptors (ssts), with IC_{50} values of 2.6 ± 0.4 nM (sst_1), 0.9 ± 0.1 nM (sst_2), 1.5 ± 0.2 nM (sst_3), 1.6 ± 0.1 nM (sst_4), 0.65 ± 0.1 nM (sst_5) (Reubi et al., 2002), while SOM230 has high affinity to 4 of the 5 ssts, with IC_{50} values of 9.3 nM (sst_1), 1.0 nM (sst_2), 1.5 nM (sst_3), >1000 nM (sst_4), 0.16 nM (sst_5) (Lewis et al., 2003). It is expected that these compounds would better mimic the effects of natural somatostatins than octreotide, that displays respective IC_{50} values of >10,000 nM (sst_1), 2.0 ± 0.7 nM (sst_2), 187 ± 55 nM (sst_3), >10,000 nM (sst_4), and 22 ± 6 nM (sst_5) (Reubi et al., 2000). SOM230 for instance is being developed to treat neuroendocrine tumor patients, in particular those that have been poorly sensitive to octreotide treatment (Schmid, 2008). The rationale and the advantage of SOM230 action would be to act not only on somatostatin sst_2 receptors but primarily on the non-sst_2 receptors expressed in these tumors. Tumors of interest include somatostatin sst_5 receptor-expressing adrenocorticotropic hormone (ACTH)-producing pituitary tumors, sst_2/sst_5 or sst_5-expressing growth hormone (GH)-producing pituitary adenomas, and octreotide-resistant carcinoids (Hofland et al., 2005).

Recent *in vitro* data published on KE108 and SOM230 suggest that these compounds do not appear to simply mimic natural somatostatins in selected signal transduction pathways (Ben-Shlomo et al., 2009; Cescato et al., 2009; Lesche et al., 2009; Liu et al., 2005) but to have biased agonistic or functional selectivity properties. While they mimic natural somatostatins in specific signaling systems such as

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doi:10.1016/j.ejphar.2010.07.005

the inhibition of cAMP production, they were found to have distinct behavior in others. For instance, neither KE108 nor SOM230 have agonistic properties to stimulate intracellular calcium mobilization and ERK phosphorylation; as opposed to somatostatin or octreotide analogs, they show no effect when applied alone but completely antagonize the somatostatin or octreotide analogs effect, both in sst_2-expressing HEK293 cells and in AR42J cells endogenously expressing sst_2 (Cescato et al., 2009).

Among the clinically relevant properties of somatostatin analogs, agonist-triggered sst_2 internalization has been shown to be a potent mechanism to actively transport somatostatin radioligands into tumor cells (Cescato et al., 2006; Liu et al., 2005; Waser et al., 2009). This is relevant for scintigraphic tumor diagnosis and also for targeted tumor radiotherapy. It may therefore be of particular interest to know whether multi-somatostatin analogs such as SOM230 or KE108 are as efficiently internalized *in vivo* as [Tyr^3–Thr^8]-octreotide (TATE), in view of a potential development of radiolabeled multi-somatostatin analogs for diagnostic or radiotherapeutic purposes. It may also be worth understanding sst_2 trafficking triggered by these drugs in order to evaluate their capability of desensitization and escape during long-term treatment in tumor patients. In principle, a compound that does not lead to desensitization would have advantages over a desensitizing agonist.

We have recently developed a method permitting to evaluate the *in vivo* internalization of sst_2 in transplanted AR42J tumor cells and pancreatic cells after i.v. injection of the octreotide analog TATE (Waser et al., 2009). The aim of the present study was to use this method to investigate the ability of SOM230 to trigger sst_2 internalization *in vivo* in comparison to TATE. Rats bearing AR42J tumors endogenously expressing the somatostatin sst_2 receptors were injected intravenously with SOM230; they were euthanized at various time points and the tumors and pancreas were analyzed by immunohistochemistry for the cellular localization of the somatostatin sst_2 receptors. In addition, and for comparison, the sst_2 internalization triggered by SOM230 was also evaluated *in vitro* by immunofluorescence microscopy with the same AR42J cell line used for the *in vivo* studies.

2. Material and methods

2.1. Reagents

All reagents were of the best grade available and were purchased from common suppliers. The sst_2-specific antibody R2-88 was provided by Dr. Agnes Schonbrunn (Houston, TX, USA) and the rabbit monoclonal antibody UMB-1 (SS-8000RM) was purchased from Biotrend GmbH, Germany. The secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (H + L) was from Molecular Probes, Inc. (Eugene, OR, USA). [Tyr^3–Thr^8]-octreotide (TATE) was provided by Dr. H.R. Mäcke (Basel, Switzerland). SOM230 (pasireotide) and the two sst_2 antagonists DOTA-Bass (DOTA-[4-NO_2–Phe-c(DCys–Tyr–DTrp–Lys–Thr–Cys)-DTyr–NH_2]) (Ginj et al., 2006) and BIM-23A180 (Cpa-c(DCys–Tyr–DTrp–NMeLys–Thr–Cys)-Nal–NH_2) (Rajeswaran et al., 2001) were provided by Dr. J.E. Rivier (La Jolla, CA, USA). The purity of the compounds was >95%.

2.2. Cell line

The rat pancreatic tumor cell line AR42J (CRL-1492) was obtained from ATCC (LGC Standards, Teddington, Middlesex, UK) and cultured at 37 °C and 5% CO_2 in Ham's F12K containing 2 mM L-glutamine and supplemented with 20% (vol/vol) fbs, 100 U/ml penicillin and 100 µg/ml streptomycin. All culture reagents were from Gibco BRL, Life Technologies (Grand Island, NY).

2.3. Receptor autoradiography

Cell membrane pellets of AR42J cells were prepared and receptor autoradiography was performed on 20 µm thick pellet sections (mounted on microscope slides), as described in detail previously for pellets from other cell lines (Cescato et al., 2008; Erchegyi et al., 2009). For each of the tested compounds, complete displacement experiments were performed with the universal somatostatin radioligand [^{125}I]-[Leu^8, D-Trp^{22}, Tyr^{25}]-somatostatin-28 (2000 Ci/mmol; Anawa, Wangen, Switzerland) using 6000 cpm/100µl and increasing concentrations of the unlabeled compounds ranging from 0.1 to 1000 nmol/l. Somatostatin-28 was run in parallel as control using the same increasing concentrations. The slides were exposed to Biomax MR film (Kodak) for 7 days at 4 °C. IC_{50} values were calculated after quantification of the data using a computer-assisted image processing system (Cescato et al., 2008). Tissue standards containing known amounts of isotopes, cross-calibrated to tissue-equivalent ligand concentrations, were used for quantification (Cescato et al., 2008).

2.4. Animal tumor models

Animals were kept, treated, and cared for in compliance with the guidelines of the Swiss regulations (approval 789). A total of 10 to 12 million AR42J cells, freshly suspended in sterile phosphate-buffered saline (PBS), were subcutaneously implanted in one flank of Lewis rats (49–55 g). 11–15 days after inoculation, the rats, weighing 120–150 g, showed solid palpable tumor masses (tumor weight 70–150 mg) as reported previously (Ginj et al., 2008; Waser et al., 2009) and were used for the *in vivo* internalization experiments. Peptides were solubilized in sodium chloride solution (0.9%, 0.1% bovine serum albumin) and injected into the rats in a total volume of 0.2 ml under isoflurane anesthesia. Two rats were used for each experimental condition. A first set of rats was injected with 0.21 mg/animal SOM230 into the lateral tail vein and euthanized 2.5 min, 10 min, 1 h, 6 h and 24 h after injection. As a positive control for somatostatin sst_2 receptor internalization, 0.21 mg/animal TATE was injected in rats that where then euthanized after 1 h. Untreated rats injected only with PBS were used as negative controls. To test for antagonism a second set of rats was first injected with SOM230 (a 100-fold excess compared to the amount of TATE) into the lateral tail vein, followed 5 min later by a second injection with 0.0021 mg/animal TATE again into the lateral tail vein. This 0.0021 mg/animal low dose of TATE, still able to induce a complete sst_2 internalization (Waser et al., 2009), was used to permit the use of a lower dose of SOM230 (but still in 100 times excess of TATE) in the antagonist test. As positive control for antagonism, the sst_2 antagonist DOTA-Bass (Ginj et al., 2006), was applied instead of SOM230 in a similar experimental setting in 100-fold excess compared to TATE. The animals were then euthanized 1 h after the second injection. The application of 0.0021 mg/animal of TATE alone was used as positive control for agonism. The tumors and pancreas of each animal were collected. All samples were cut in half. One half of the samples were immersed in a 4% formalin solution for 24 h and paraffin-embedded for immunohistochemical investigations. The other half was frozen in dry ice and stored at −80 °C for further *in vitro* receptor autoradiography.

2.5. Immunohistochemistry of somatostatin sst_2 receptors

Sst_2 immunohistochemistry was performed as described before (Waser et al., 2009). The samples were either tested with the sst_2-specific polyclonal antibodies R2-88 or the rabbit monoclonal antibody UMB-1 (Fischer et al., 2008; Korner et al., 2005). Formalin-fixed, paraffin-embedded tissue sections (4 µm-thick) were used. The best antigen-retrieval method for R2-88 and UMB-1 immunohistochemistry was boiling in the microwave in 5% urea buffer (pH 9.5). R2-88 was applied in a 1:1000 dilution and UMB-1 in a 1:100 dilution. The secondary antibody was a biotinylated goat anti-rabbit