Somatostatin receptor sst2 gene transfer in human prolactinomas in vitro: Impact on sensitivity to dopamine, somatostatin and dopastatin, in the control of prolactin secretion

Thomas Cuny a,b, Amira Mohamed a, Thomas Graillon a,c, Catherine Roche a, Céline Defilles a, Anne-Laure Germanetti d, Bettina Couderc e, Dominique Figarella-Branger f, Alain Enjalbert a,d, Anne Barlier a,d, Alexandru Saveanu a,d,*

a Aix-Marseille University, CRN2M, UMR 7286-CNRS, 51, Bd Pierre Dramard, 13344 Marseille, France
b University Hospital of Nancy, Department of Endocrinology, Allée de Morvan, 54500 Vandoeuvre-Les-Nancy, France
c AP-HM, Department of Molecular Biology, CHU Conception – 147 Bd Baile, 13385 Marseille, France
d AP-HM, Laboratory of Molecular Biology, CHU Conception – 147 Bd Baile, 13385 Marseille, France
e CLCC, Institut Claudius Regaud – EA3035, 20–24 Rue du Pont Saint-Pierre, 31052 Toulouse, France
f AP-HM, Department of Anatomopathology, CHU Timone, 264, Rue Saint-Pierre, 13385 Marseille, France

**Corresponding author. Address:** Aix Marseille University, CRN2M UMR 7286 CNRS, Faculté de Médecine – Secteur Nord – CS8001, 51, Bd Pierre Dramard, 13344 Marseille, Cedex 13, France. Tel.: +33 491 69 89 17; fax: +33 491 69 89 20. E-mail addresses: t.cuny@chu-nancy.fr (T. Cuny), alexandru.saveanu@univ-amu.fr, alexandru.saveanu@univ-med.fr (A. Saveanu).

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**ABSTRACT**

**Objective:** As prolactinomas fail to respond to dopamine agonist (DA) in 10–20% of cases, we hypothesized that somatostatin subtype 2 receptor (sst2) overexpression in DA-resistant prolactinomas may enhance suppression of prolactine (PRL) using chimeric agonist (dopastatin) that simultaneously binds sst2 and the dopamine subtype 2 receptor (D2DR).

**Design and methods:** PRL suppression by octreotide, sst5 agonist, sst2-D2DR agonist (BIM-23A760 dopastatin) and cabergoline was assessed in primary cultures of seven DA-resistant prolactinomas overexpressing sst2.

**Results:** sst2 was effectively overexpressed via adenoviral expression in prolactinomas (38.1 ± 7.4 vs. 0.1 ± 0.1 copy/copy β-Gus) and induced octreotide sst2-mediated PRL suppression that remained lower than that induced by DA. BIM-23A760 inhibited PRL similarly to cabergoline both in the control and sst2-expressing cells. Antagonist experiments confirmed predominant dopaminergic effect in dopastatin activity.

**Conclusion:** sst2 was successfully overexpressed in prolactinomas. However BIM-23A760 was unable to enhance PRL suppression underlining a predominant dopaminergic contribution in its action.

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1. Introduction

Prolactinomas are the most frequent type of pituitary adenomas representing 45–65% of cases (Klibanski, 2010). Dopamine agonists (DA), such as cabergoline, are the treatment of choice to control prolactin (PRL) secretion and cell proliferation in these benign tumors (Colao et al., 2002). The pharmacologic action of DA is mediated by dopamine receptor subtype 2 (D2DR) which is expressed at the lactotroph cell membrane (Missale et al., 1998). Nevertheless, between 10% and 20% of prolactinomas are resistant to DA, defined as failure to normalize PRL levels and reduce tumor size (Melmed et al., 2011; Molitch, 2005). This molecular resistance is, at least partially, explained by lower expression of D2DR mRNA and corresponding reduced protein levels in resistant adenomas compared to sensitive ones (Caccavelli et al., 1994). Resistant prolactinomas present as classic large and/or invasive tumors, making surgical removal difficult and radiotherapy approaches, with subsequent risk of hypopituitarism, complicated (Delgrange et al., 2009; Fernandez et al., 2009). Thus, the emergence of alternative medical options is a real challenge for these problematic cases.

D2DR is expressed not only in prolactinomas, but also in all other pituitary adenomas, and its expression in pituitary cells is always associated with one or more of the five somatostatin receptor subtypes (sst1–5) (Ben-Shlomo and Melmed, 2010; Stefaneanu et al., 2001). Interestingly, sst and D2DR belong to the same G-protein-coupled receptor (GPCR) family, where cooperation through GPCR heterodimerization has already been extensively described, and may enhance agonist binding, transduction pathway activation
or receptor turnover (Bulenger et al., 2005; Duran-Prado et al., 2008). For example, agonist-induced heterodimerization of D2DR and sst2 have been recently described in a model of transfected cells as well as in neuronal cells, upon treatment with selective ligands, and was accompanied by increased affinity for dopamine and augmented D2DR signaling as well as prolonged sst2 internalization (Baragli et al., 2007).

The best interaction model in the pituitary to gauge putative sst-D2DR interactions are growth-hormone (GH) secreting-adenomas as they express significant amounts of functional sst2, sst5 and to a lesser extent, D2DR (Ben-Shlomo and Melmed, 2010; Stefaneanu et al., 2001; Saveanu et al., 2008). Indeed, in GH-adenomas, DA effectively controls GH secretion in approximately 30% cases (Moyes et al., 2008), while current somatostatin analogs (octreotide, lanreotide), primarily targeting sst2, control GH secretion and proliferation of GH-adenomas in about 60% of cases (Colao et al., 2011). Inoctreotide-resistant GH-adenomas in cell culture, we showed that sst2-D2DR chimeric agonists, known as dopastasins, are able to enhance GH suppression in comparison to either sst2 or D2DR agonists alone or in combination, by acting on both sst2 and D2DR receptors (Saveanu et al., 2002).

In prolactinomas, sst expression profiles are substantially different; the dominant sst subtypes are sst5 and sst1, while sst2 expression is low (Fusco et al., 2008; Jaquet et al., 1999). We previously showed in prolactinoma cell cultures that sst5 agonists were more effective than sst2, including octreotide, in suppressing PRL secretion but were not more effective than DA (Fusco et al., 2008; Jaquet et al., 1999). Unlike GH-adenomas, the sst2-D2DR chimeric agonist had a roughly similar effect on PRL secretion to that obtained with a D2DR agonist (cabergoline).

In octreotide-resistant GH-adenomas, we recently showed that enhancing sst2 expression by sst2 adenoviral gene transfer in vitro restored effective octreotide-mediated suppression of GH secretion (Acunzo et al., 2008). In the same study, in two prolactinomas, sst2 overexpression was associated with a significant suppression of PRL by octreotide which was very similar to that obtained with cabergoline. Therefore, our aim was to further analyze the effect of sst2 overexpression in a group of seven human prolactinomas. As DA-resistant prolactinomas still have a higher expression of D2DR than GH-adenomas (Fusco et al., 2008), we postulated that in sst2-overexpressing prolactinoma cell cultures, sst2 and D2DR coexpression might create a good model to test the efficacy of the chimeric sst2-D2DR agonist, BIM-23A760, on PRL suppression (Jaquet et al., 2005).

2. Design and methods

2.1. Patients

The present study was approved by the Ethics Committee of the University of Aix-Marseille II (Aix-Marseille, France) and informed consent was obtained from each patient. Seven patients with PRL-secreting pituitary adenomas were included in the study (two men, five women, Table 1). The endocrine and neuroradiological characteristics of tumors were documented before any treatment. All patients were considered as resistant in vivo to dopamine agonist therapy (no normalization of PRL secretion and/or significant shrinkage of tumor size until 3.5 mg/week of cabergoline) (Molitch, 2005) and then underwent transsphenoidal surgery in the Neurosurgery Department. Tumor fragments were subjected to anatomopathology and molecular biology laboratory analysis. In all cases, anatomopathology confirmed the PRL phenotype by immunohistochemistry. Tumor fragments were used for cell culture studies and determination of mRNA expression of D2DR, sst1, sst2, and sst5.

2.2. Cell culture

Tumor fragments obtained from transsphenoidal surgery were submitted to mechanical and enzymatic dissociation with collagenase at 37 °C for 60 min. Total cell amounts were 4 × 10⁶ to 65 × 10⁶, depending on the tumor. For hormonal assays, tumor cells were plated at a density of 1.5 × 10⁴ in 24-well cultures dishes coated with extra cellular matrix (ECM) for bovine endothelial corneal cells, as previously described (Jaquet et al., 1985). For cyclic AMP (cAMP) studies, 3 × 10⁴ adenoma cells were plated in 24-well dishes. For FACS (Fluorescence-Activity Cell Sorting) and RNA extraction, 10⁴ adenoma cells were plated in 6-well dishes. For immunocytochemistry, 10⁴ cells were plated on ECM-coated 14-mm cover glass and the cells were then cultured for 3 days at 37 °C in a CO₂ incubator with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, streptomycin (100 U/ml) and glutamine (100 U/ml).

2.3. Cell infection

The sst2 gene was introduced using an adenoviral vector as previously described (Acunzo et al., 2008). Briefly, after 72 h of culture at 37 °C and 5% CO₂, cells were infected with an adenoviral vector containing either enhanced green fluorescent protein (eGFP) gene alone (subsequently referred to as “Ad-eGFP”), or both eGFP and mouse sst2 gene (subsequently referred to as “Ad-SST2”). In all experiments, Ad-SST2 infected cells were compared to Ad-eGFP infected cells. Viral infections were done using a multiplicity of infection of 5 (SMOI), a viral dose that previously showed high-level transgenic expression and no effect on the viability of human pituitary cells (Acunzo et al., 2008). Three hours after infection, the viral suspension was replaced by 1 ml of complete culture medium in infected as well as in non-infected wells.

2.4. Validation of infection

2.4.1. Flow cytometry analysis

To determine transduction efficacy, 2.5 × 10⁵ control or Ad-SST2 cells were detached from the matrix by enzymatic methods (trypsin) four days after infection, collected in tubes containing

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Tumor size (mm)</th>
<th>Basal PRL (ng/mL)</th>
<th>PRL under DA therapy (ng/mL)</th>
<th>Decrease in tumor size (&gt;50%) under DA therapy</th>
<th>IHC</th>
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<td>30</td>
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<td>35</td>
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<td>P3</td>
<td>18</td>
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<td>No</td>
<td>PRL: 100%</td>
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<td>P4</td>
<td>13</td>
<td>F</td>
<td>27</td>
<td>6000</td>
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<tr>
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<td>F</td>
<td>10</td>
<td>565</td>
<td>289</td>
<td>No</td>
<td>PRL: 100%</td>
</tr>
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Table 1: Clinical and immunohistochemical characteristics of 7 human DA-resistant prolactinomas. IHC: immunohistochemistry. DA = dopamine analog (cabergoline). DA therapy was increased till 3.5 mg/week cabergoline.
800 µl of complete culture medium with propidium iodide (PI; 0.5 µg/ml; BD Pharmingen, San Diego, California) and subjected to FACS analysis (Becton Dickinson). Data were analyzed with the Cell quest program (Becton Dickinson). Ten thousand events were acquired for each analysis and assayed in duplicate.

### 2.4.2. RNA extraction and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Three days after infection, total mRNAs were extracted using the RNeasyMicrokit (Qiagen, Hilden, Germany). One microgram of total RNA was used for cDNA synthesis during reverse transcription, as previously described (Jaquet et al., 1999). D2DR, sst1, sst2 and sst5mRNAs were detected by real time quantitative PCR using specific primer and probes as previously described (Jaquet et al., 1999). The mRNA levels of each receptor subtype were normalized to the beta-glucuronidase (β-Gus) mRNA level, as previously described (Saveanu et al., 2002).

### 2.4.3. Immunocytochemistry

Three days after infection, the expression and localization of sst2 and D2DR were assessed by immunocytochemistry in one tumor (P5). After fixing, cells were incubated overnight at 4 °C with an antisera against sst2A (SS-800; Gramsch Lab, Germany) diluted at 1:1000 in PBS supplemented with 1% bovine serum albumin (sigma). The immunostaining was visualized using Alexa 594-conjugated goat anti-rabbit IgG (Molecular Probes, Invitrogen) diluted at 1:800 in PBS containing 10% normal goat serum. The nucleus of each cell was visualized through DAPI counterstain (in blue). Confocal image acquisition was performed on a Leica TCS SP2 laser scanning microscope.

The mouse monoclonal antibody SC-5303 (Santa Cruz Biotechnology, Inc.) was used to detect D2DR. Cells were incubated overnight at 4 °C with the antisera against D2DR diluted at 1:100 in PBS supplemented with 1% bovine serum albumin. Secondary antibody and image acquisition were then performed as described for sst2.

### 2.4.7. Cyclic adenosine monophosphate (cAMP) assay

One of the main transduction pathways of Ga-protein-coupled receptors, such as sst2 and D2DR, is suppression of cAMP synthesis (Missale et al., 1998; Ben-Shlomo and Melmed, 2010). We assessed the effect of different agonists (sst2, D2DR, sst2-D2DR) on forskolin-stimulated cAMP production in three prolactinomas (P2, P5, P7). The cAMP concentration was determined by HTRF-cAMP femto kit based on a competitive immunoassay using cryptate antibody labeled anti-cAMP and the XL665-labeled cAMP (CisBio International, Bagnols-sur-Cèze, France).

Four days after infection, cells were detached from the matrix, placed in 96-wells plates, then incubated at 37 °C for 3 h in the presence of 1 Mol/L of forskolin alone (control wells) or combined with 10 nMol/L octreotide, 0.1 nMol/L cabergoline or 0.1 nMol/L BIM-23A760. The HTRF assay reagents were then added and left for 1 h at room temperature, as described by the manufacturer. Time-resolved FRET signals were measured 50 µs after excitation at 620 and 665 nm using a Mithras multiplex reader (Berthold Technologies, France). cAMP standard concentrations provided by the manufacturer were assayed at the same time, allowing interpolation of sample cAMP concentrations (nMol/L). Results are expressed as percentage cAMP vs. control. Each experimental condition was assayed in triplicate.

### 2.8. Statistical analysis

Results are presented as the mean ± SEM. Statistical significance between two unpaired groups was determined by the Mann–Whitney U test. To measure the strength of association between the pairs of variables without specifying dependencies, Spearman rank correlations were used. A p-value <0.05 was considered significant for all tests.

### 3. Results

#### 3.1. Adenoviral transduction efficiency and sst/D2DR expression

Three days after infection, the mean percentage of eGFP-expressing cells was 71 ± 8% and 70 ± 10%, respectively in Ad-eGFP and Ad-SST2 cells (data not shown), indicating effective adenoviral infection in cultured prolactinoma cells. Besides infected-conditions we also tested non-infected prolactinoma cells where eGFP expression was, as expected, no detected.

Sst1, sst2, sst5 and D2DR mRNA were expressed in all seven Ad-eGFP prolactinomas. As expected, D2DR mRNA levels were higher (51.6 ± 35 copy/copy βGus) than sst mRNA in all cases (sst5 0.4 ± 0.24 and sst1 0.73 ± 0.4 copy/copy βGus, respectively). The mean sst2 mRNA level was markedly lower (0.1 ± 0.4 copy/copy βGus) than sst1 and sst5. In all Ad-SST2 prolactinomas, sst2 mRNA
levels had dramatically increased (38.1 ± 7.4 copy/copy β-Gus, p < 0.05 vs. Ad-eGFP cells) without significantly modifying the mean level of D2DR (42.3 ± 25.4 copy/copy β-Gus in Ad-SST2 cells), suggesting that sst2 induction was successful and that increased levels of sst2 do not affect D2DR mRNA expression. The increase in sst2 mRNA did not modify sst1 or sst5 mRNA expression neither (Fig. 1).

In Ad-SST2 cells, the mean level of sst2 mRNA was roughly similar to the level of D2DR mRNA (sst2 mRNA/D2DR mRNA = 0.9 ± 0.3).

Seventy-two hours after infection, immunocytochemistry analysis showed weak and mainly cytoplasmic sst2 staining in Ad-eGFP cells (Fig. 2A). In Ad-SST2 cells, the sst2 immunostaining was clearly stronger and located mainly at the membrane (Fig. 2B). Immunocytochemistry analysis of D2DR was roughly similar in both cell types with D2DR located primarily at the membrane level (Fig. 2C and D), signifying that increased levels of sst2 expression do not affect both D2DR levels and localization.

3.2. Effect of the D2DR, sst, and sst2-D2DR agonists on PRL secretion in Ad-eGFP prolactinoma cells

The D2DR agonist cabergoline, produced a dose-dependent inhibition of PRL release with a mean maximal percentage of PRL suppression of 59 ± 6.7% (EC50 = 9 × 10^{-11} M/L) in all seven prolactinomas (Fig. 3A). As expected, octreotide was ineffective on PRL secretion until 10 nMol/L (7 ± 2.2%) (Fig. 3A), whereas the sst5 preferential agonist, BN-82087, induced a significantly stronger inhibition of PRL release in all seven control tumors (mean % of decrease: 28 ± 2.8%) at a concentration of 10 nMol/L (Fig. 3D, left panel).

BIM-23A760 induced a dose-dependent inhibition of PRL secretion similar to that obtained under cabergoline (mean maximal inhibition of PRL secretion = 58.7 ± 6.8%, EC50 = 3 × 10^{-12} M/L) (Fig. 3B).

3.3. Effect of D2DR, sst and sst2-D2DR agonists on PRL secretion in Ad-SST2 prolactinoma cells

Sst2 overexpression in prolactinoma cells was associated with a dose-dependent suppression of PRL release by octreotide in all seven tested tumors, significant from 10^{-10} to 10^{-8} M/L (Fig. 3A).

Maximal mean PRL suppression induced by octreotide was 28 ± 3.4% at 1 and 10 nMol concentrations and was markedly higher than in Ad-eGFP cells (p < 0.001).

Surprisingly, sst2 overexpression did not improve the sensitivity to BIM-23A760 of prolactinoma cells (EC50 = 2 × 10^{-11} M/L): a similar dose-dependent profile of PRL suppression was observed in Ad-eGFP and Ad-SST2 cells (Fig. 3B). The dose dependent inhibition of PRL secretion under cabergoline was similarly unaffected by sst2 overexpression (EC50 = 10^{-11} M/L), (Fig. 3C). Both cabergoline and BIM-23A760 exhibited the same mean maximal inhibitory effect on PRL suppression (58.5 ± 7% and 59.5 ± 3.1%, respectively) in Ad-SST2 cells and in Ad-eGFP cells (Fig. 3B and C). Nevertheless, the EC50 under BIM-23A760 was slightly (but not significantly) lower than that achieved with cabergoline in both conditions. Note that the maximal inhibition obtained in Ad-SST2 cells with cabergoline or BIM-23A760 remained stronger than that obtained with octreotide (p < 0.001, Fig. 3A–C).

Similarly, sst2 overexpression did not modify the effect of the sst5 agonist, which achieved a mean maximal inhibition of PRL secretion of 27.5 ± 2.6% (Fig. 3D, right panel), similar to that obtained in Ad-eGFP cells.

In conclusion, the inhibitory effects of octreotide on PRL secretion in Ad-SST2 prolactinoma cells (Fig. 3A) equaled those of sst5 agonist (Fig. 3B) but represented only half of the maximal inhibitory effect obtained with cabergoline or BIM-23A760.

3.4. Effect of drugs on forskolin-induced cAMP production

In Ad-eGFP cells, octreotide at 10 nMol/L concentration, was unable to suppress FSK-stimulated cAMP production, while cabergoline and BIM-23A760 at 0.1 nMol/L concentration induced a similar and significant suppression of cAMP production (70 ± 6.3% and 67 ± 2.7%, respectively, p < 0.001 both vs. control) in three prolactinomas (Fig. 4).

In Ad-SST2 cells, octreotide achieved a significant suppression of cAMP at 10 nMol/L concentration (39 ± 1.4%, p < 0.01 vs. control), while cAMP suppression by cabergoline and BIM-23A760 was, once again, similar and not significantly different from that obtained in Ad-eGFP cells (79 ± 7.3% and 79 ± 5.6%, respectively). As for PRL secretion, in Ad-SST2 cells, octreotide was less effective than either D2DR or sst2-D2DR agonist in suppressing cAMP production (p < 0.01).

3.5. Effect of sst2 and D2DR antagonists

As BIM-23A760 did not achieve better suppression of PRL in Ad-SST2 in comparison to control cells, we assessed the contribution of sst2 and D2DR moieties in PRL suppression, by using specific sst2 and D2DR antagonists (BIM-23454 and Sulpiride, respectively) in three tumors (P1, P4 and P6). In Ad-eGFP cells, sulpiride completely reversed the PRL suppression induced by both cabergoline and BIM-23A760 effects (0.1 nMol/L, p < 0.05), while BIM-23454 was ineffective on BIM-23A760 action (Fig. 5A). In Ad-SST2 cells, sulpiride completely reversed the effect of cabergoline and almost completely abrogated that of BIM-23A760, suggesting dominant D2DR-mediated PRL suppression by BIM-23A760 (p < 0.05, Fig. 5B). The sst2 antagonist, BIM-23454, was effective in reversing octreotide-induced (10 nMol/L) PRL suppression in Ad-SST2 cells suggesting sst2-mediated PRL suppression by octreotide (Fig. 5B; 25 ± 4% under octreotide vs. 3 ± 2% under octreotide and BIM-23454; p < 0.05). However, sst2 antagonists had no significant effects on BIM-23A760-induced PRL suppression, confirming little or no contribution of the sst2 moiety of BIM-23A760 in PRL suppression (Fig. 5B; 44 ± 4% under BIM-23A760 vs. 46 ± 5% under BIM-23A760 and BIM-23454; NS).
4. Discussion

We hypothesized that overexpression of sst2 in DA-resistant prolactinomas by adenoviral transfer would create an optimal cell system for an sst2-D2DR chimeric agonist, dopastatin, action.

Our results show that sst2 overexpression in prolactinomas considered in vivo as resistant to dopamine agonist therapy, was effective and that an sst2 agonist became able to suppress PRL secretion. However, BIM-23A760, a last generation dopastatin, was not able to trigger sst2-D2DR additivity either on PRL secretion or cAMP suppression.

In basal conditions, we previously showed that BIM-23A760 exhibits similar effects to cabergoline, both in DA-sensitive and DA-resistant tumors (Fusco et al., 2008). This was explained by the low level of sst2 mRNA expression in tumoral lactotroph cells and subsequently a predominant or exclusive dopaminergic effect of the dopastatin. In other types of pituitary adenomas presenting low sst2 expression, such as Non-Functioning Pituitary Adenomas (NFPAs) or ACTH-secreting adenomas, dopastatin also showed an efficacy only equivalent to D2DR agonist (Florio et al., 2008; Hofland et al., 2010). In contrast, in GH-adenoma cells that were partially sensitive to octreotide, we previously observed in vitro, an enhanced efficacy on GH suppression of a first generation dopastatin (BIM-23A387) as compared to either sst2 or D2DR agonists alone or combined (Saveanu et al., 2002). This sst2-D2DR cooperation may be related to ligand-induced receptor heterodimerization, as shown in CHO-K1 cells stably co-transfected with sst2 and D2DR (Baragli et al., 2007). Agonist-induced heterodimerization was associated with increased affinity for D2DR agonist and increased D2DR signaling as well as prolonged sst2 internalization, suggesting a functional cooperation between D2DR and sst2 (Baragli et al., 2007).

In our seven prolactinomas, sst2 transfer was effective and the mean level of sst2 mRNA expression became roughly similar to the level of D2DR mRNA of these cells, resulting in high levels of both sst2 and D2DR mRNA, higher than that found in GH-adenomas. Moreover, immunocytochemistry also showed strong membrane immunostaining for both sst2 and D2DR in most Ad-SST2 cells, then creating favorable conditions for hypothetic cooperation. However, the BIM-23A760 effects on PRL suppression were less than expected, as no significant improvement was evidenced on maximal PRL suppression or EC50, as compared to control conditions. In both conditions, PRL suppression induced by BIM-23A760 was similar to that induced by the D2DR agonist, cabergoline. Antagonist experiments confirmed the predominance of the dopaminergic effect on BIM-23A760 action in Ad-SST2 prolactinoma cells. Indeed, while in GH-adenomas BIM-23A387 dopastatin effect at submaximal concentration was similarly reversed by sst2 and D2DR antagonists (Saveanu et al., 2002), in Ad-SST2 prolactinoma cells, D2DR antagonist alone was able to reverse BIM-23A760-induced PRL suppression and sst2 antagonist had no effect. These results seem

![Fig. 2. Confocal microscopy sections (63×) of sst2 and D2DR immunocytochemistry of one human prolactinoma (P5). Sst2 (red emission) analysis in Ad-eGFP (A) and in Ad-SST2 cells (B). D2DR (red emission) analysis in Ad-eGFP (C) and in Ad-SST2 cells (D). Immunocytochemical analysis was done 96 h after adenoviral infection. The nuclei were in blue due to DAPI coloration. Scale bars, 10 μm.](image-url)
contradictory, but there is some evidence suggesting a predominant D2DR activity for the BIM-23A760 dopastatin. Indeed, in HEK sst2 and D2DR, transfected cell lines, potency of BIM-23A760 on sst2-mediated cAMP suppression seems less than expected by its binding affinity, while D2DR mediated cAMP suppression is greater than expected by its binding affinity (Bruin et al., 2008). Moreover, cAMP suppression in sst2 and D2DR co-transfected cells was similar with either dopastatin or sst2 or D2DR-preferential agonists. So, one explanation may come from the BIM-23A760 activity profile. Another may be the singular feature of DA-resistant prolactinomas. Indeed, D2DR receptor expression levels are lower in resistant prolactinomas compared to sensitive ones (Caccavelli et al., 1994), and are also associated with molecular abnormalities of signal transduction pathways downstream of D2DR (Caccavelli et al., 1996). As shown for sst5-D2DR cooperation (Jaquet et al., 1999), it is possible that sst2-D2DR interaction cannot increase the maximal PRL suppression effect because of defects in common transduction pathways further down in the signaling cascade.

We looked primarily at the main transduction pathway linked to Gαi-coupled receptors, sst2 and D2DR, and shown to be modified by sst2-D2DR cooperation (Baragli et al., 2007) i.e. the agonist-induced cAMP suppression. Our results show that BIM-23A760 does not enhance cAMP suppression in Ad-SST2 cells, suggesting that putative sst2-D2DR cooperation may already be limited at the level of Gα protein present in the cellular pool. This is supported by the fact that DA-resistant prolactinomas present lower levels of Gα protein mRNA (Caccavelli et al., 1996).

Fig. 3. (A)–(C) Mean dose–response PRL suppression obtained in cell cultures from 7 human prolactinomas infected by 5MOI Ad-eGFP (solid lines) and Ad-SST2 (dotted lines) with the following drugs: A. Octreotide (10^−13 to 10^−8 Mol/L). ***P < 0.01, ****P < 0.001 Ad-SST2 vs. Ad-eGFP. (B) BIM-23A760 (10^−13 to 10^−8 Mol/L). (C) Cabergoline (10^−13 to 10^−8 Mol/L). (D) Mean PRL suppression obtained in 5 MOI Ad-eGFP (white bars) and Ad-SST2 (gray bars) under 10^−8 Mol/L sst5 agonist, BN-82087. ***P < 0.001 vs. control; NS = not significant Ad-SST2 vs. Ad-eGFP. Results are expressed as mean ± SEM percentage of PRL suppression vs. control (medium alone in Ad-eGFP or Ad-SST2, respectively). Each experimental condition was done in triplicate.

Fig. 4. Suppression of forskolin-stimulated cAMP synthesis assessed in cell cultures of 3 human prolactinomas (P2, P5, and P7), 4 days after infection by 5 MOI Ad-eGFP (white bars) or Ad-SST2 (grey bars), by octreotide (10 nMol/L), BIM-23A760 (0.1 nMol/L) and cabergoline (0.1 nMol/L). Results are expressed as mean ± SEM percentage of cAMP inhibition vs. control (medium alone in Ad-eGFP and Ad-SST2, respectively). Each experimental condition was done in triplicate. **P < 0.01; NS = not significant.
Octreotide, a preferential sst2 agonist, is weakly effective on PRL suppression both *in vivo* and *in vitro* (Fusco et al., 2008; Jaquet et al., 1999; Lamberts et al., 1986). Our study confirms that octreotide, ineffective at suppressing PRL secretion in basal conditions (Jaquet et al., 1999), induces a significant inhibition of hormonal secretion in all sst2-overexpressing prolactinoma cells (Acunzo et al., 2008). Octreotide-induced PRL suppression is clearly less effective than the D2DR agonist, cabergoline, in all seven Ad-SST2 tumor cells. It seems that sst2 cannot realize a coupling with pathways inducing PRL suppression as efficient as D2DR, and this was observed as early as cAMP suppression. An explanation may be that D2DR expression in our prolactinomas is still high and may sequester G_i proteins. Indeed recently it was shown that D2DR homodimers form functional units with G_i protein even in absence of agonist (Han et al., 2009).

Nevertheless, the maximal PRL suppression achieved by octreotide in Ad-SST2 cells was similar to that obtained with a highly specific sst5 agonist in both Ad-eGFP and Ad-SST2 cells. In our experiments, sst2 antagonist completely reversed octreotide-induced PRL suppression of Ad-SST2 cells, suggesting that it is octreotide’s affinity for sst2, rather than its lower affinity for sst5, that is responsible for suppression of PRL. Nevertheless, similar efficacy of sst2 and sst5 agonists suggests that sst2 activation by octreotide may directly or indirectly use the transduction pathways of sst5 in Ad-SST2 cells. Sst5 is the main sst subtype expressed in prolactinomas (Jaquet et al., 1999; Thodou et al., 2006) and previous studies have shown that sst5 agonists are the most effective somatostatin analogs at suppressing PRL secretion (Fusco et al., 2008; Jaquet et al., 1999), even if they are not more effective than D2DR agonists. Sst5 and sst2 are coupled to common signaling pathways in non-pituitary cell-lines (CHO or HEK 293 cells) (Ben-Shlomo and Melmed, 2010; Lahou et al., 2004) and have been shown to cooperate through heterodimerization (Grant et al., 2008). Moreover, the presence of sst5 modifies sst2 trafficking and signaling (Sharif et al.,

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**Fig. 5.** Antagonist experiments: Mean PRL suppression obtained in cell culture from 3 human prolactinomas, in Ad-eGFP (panel A) and in Ad-SST2 cells (panel B). Cells were incubated over a 16 h period with Cabergoline (0.1 nMol/L), BIM-23A760 (0.1 nMol/L), or octreotide (10 nMol/L only for Ad-SST2 cells), and either with (+) or without (−) BIM-23454 10^{-7} Mol/L (sst2 antagonist) or sulpiride 10^{-5} Mol/L (D2DR antagonist). Results are expressed as mean ± SEM percentage of PRL suppression vs. control (medium alone in Ad-eGFP (A) or Ad-SST2 (B)). Each experimental condition was done in triplicate. *P < 0.05, NS = not significant.
The sst5 transduction pathway could be activated by octreotide through either sst2–sst5 heterodimerization or cross-talk between transduction pathway downstream of the receptors. A similar cross-talk mechanism, where agonist binds one receptor while transduction is activated by the second one, has already been shown using truncated receptors for sst5–sst5 homodimers and sst1–sst5 heterodimers (Rocheville et al., 2000). Antagonist experiments using highly selective non-peptidic sst5 antagonists might be of interest to confirm such hypotheses (Martin et al., 2007).

In conclusion, we created a pituitary cellular model where sst2 and D2DR are coexpressed at similar and high levels in human pituitary cells. However, somatostatin-dopamine chimeric agonist (BIM-23A760) was unable to trigger cooperation between sst2 and D2DR in suppressing PRL secretion. Nevertheless, in ACTH adenomas, sst and D2DR cooperation could explain, at least in part, the improvement of hormonal suppression by association of cabergoline and the sst multivalent analog, pasireotide (Feelders et al., 2008). Cooperation between sst2 and D2DR in acromegaly could explain, at least in part, the improvement of hormonal suppression by association of cabergoline and pasireotide. Nevertheless, in ACTH adenomas, sst and D2DR cooperation could explain, at least in part, the improvement of hormonal suppression by association of cabergoline and pasireotide (Feelders et al., 2010). Therefore, further investigations are needed to clarify the conditions of sst2–D2DR interaction in neuroendocrine cells and the role of chimeric somatostatin-dopamine molecules.

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