Somatostatin and dopamine receptor interaction in prostate and lung cancer cell lines

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Abstract

Somatostatin analogues inhibit in vitro cell proliferation via specific membrane receptors (SSTRs). Recent studies on transfected cell lines have shown a ligand-induced formation of receptor dimers. The aim of this study is 1) to evaluate the role of specific ligands in modulating receptor interactions in the androgen-dependent prostate cancer cell line, LNCaP, and in the non-small cell lung cancer line, Calu-6, by co-immunoprecipitation and immunoblot; and 2) to correlate the antiproliferative effect of these compounds with their ability in modulating receptor interactions. In LNCaP, we have demonstrated the constitutive presence of sstr1/sstr2, sstr2/sstr5, sstr5/dopamine (DA) type 2 receptor (D2R), and sstr2/D2R dimers. BIM-23704 (sstr1- and sstr2-preferential compound) increased the co-immunoprecipitation of sstr1/sstr2 and significantly inhibited proliferation (K30.98%). BIM-23244 (sstr2–sstr5 selective agonist) significantly increased the co-immunoprecipitation of sstr2/sstr5, and induced a K41.36% inhibition of proliferation. BIM-23A760, a new somatostatin/DA chimeric agonist with a high affinity for sstr2 and D2R and a moderate affinity for sstr5, significantly increased the sstr5/D2R and sstr2/D2R complexes and was the most powerful in inhibiting proliferation (42.30%). The chimeric compound was also the most efficient in modulating receptor interaction in Calu-6, increasing the co-immunoprecipitation of D2R/sstr5 and inhibiting cell proliferation (30.54%). However, behind BIM-23A760, BIM-53097 (D2R-preferential compound) also significantly inhibited Calu-6 proliferation (17.71%), suggesting a key role for D2R in receptor cross talk and in controlling cell growth. Indeed, activation of monomeric receptors did not affect receptor co-immunoprecipitation, whereas cell proliferation was significantly inhibited when the receptors were synergistically activated. In conclusion, our data show a dynamic ligand-induced somatostatin and DA receptor interaction, which may be crucial for the antiproliferative effects of the new analogues.


Introduction

Somatostatin (or somatotropin release-inhibiting factor, SRIF) acts through five specific G-protein-coupled membrane receptors (SSTRs), code named sstr1–5, expressed in SRIF-target cells (Patel 1999, Møller et al. 2003). SRIF mainly exerts an inhibitory effect on cell functions, such as secretion and proliferation (Lamberts et al. 2002). The inhibitory activity of SRIF is replicated by the SRIF analogues octreotide and lanreotide, already used in the clinic practice to control hormonal hypersecretion by the pathological tissues highly expressing sstr2, such as GH-producing pituitary adenomas or certain neuroendocrine tumors (Weckbecker et al. 2003). The stimulation of SSTRs by SRIF analogues may also produce an antiproliferative effect in vitro, due to either cell cycle arrest or induction of apoptosis (Danila et al. 2001, Ferone et al. 2002, Ferrante et al. 2006, Florio 2008).

In the past years, the knowledge about SSTRs physiopathology has been extended by the discovery that SSTRs can interact on cell membrane forming receptor dimers (Rocheville et al. 2000a). A series of studies, carried out on transfected cell lines, have shown that dimers can consist of two identical SSTR subtypes (homodimers) or two different subtypes (heterodimers), with a range of possible combinations depending on the specific subtype and, probably, on the specific SSTR-expressing cell population. SSTR dimerization can be a ligand-dependent phenomenon, since natural SRIF and subtype-specific SRIF agonists have different effects on dimerization, leading to formation or, on the contrary, dissociation of SSTR dimers (Rocheville et al. 2000a,
SSTR homo- and heterodimerization also involve cellular events beyond the membrane, since it modifies SSTR internalization and trafficking (Baragli et al. 2007), as well as signal transduction (Kidd et al. 2008). SSTRs can also heterodimerize with other G-protein-coupled receptors, such as the μ-opioid receptor (Pfeiffer et al. 2002) and dopamine (DA) type 2 receptor (D₂R; Rocheville et al. 2000b, Baragli et al. 2007).

The heterodimerization of these G-protein-coupled receptors and the related cellular events open a new possible scenario in the field of treatments targeting SSTR-expressing tumors (Papotti et al. 2002). In fact, new SRIF analogues, which bind more than one SSTR subtype (Weckbecker et al. 2003) and chimeric compounds binding SSTRs and D₂R, have been developed and are now available for phase 2 studies (Ferone et al. 2007). Indeed, SSTRs and D₂R are frequently co-expressed in those diseases for which therapies with SRIF analogues have already been approved (O’Toole et al. 2006, Ferone et al. 2008, Srirajaskanthan et al. 2009). However, only few studies have been performed to evaluate the final consequences of G-protein-coupled receptors dimerization on cell functions so far (Ferone et al. 2005, Grant et al. 2008), and the clinical relevance of SSTR and D₂R heterodimerization must be proved.

In this study, we analyzed the effects of different SRIF analogues and of one SRIF/DA chimeric compound on SSTRs and D₂R interaction on cell membrane and cell proliferation in a cell line constitutively expressing four out of five SSTRs, the androgen-dependent prostate cancer cell line LNCaP (Ruscica et al. 2010), and in a cell line constitutively expressing three out of four SSTRs and the D₂R, the non-small cell lung cancer (NSCLC) line Calu-6 (Ferone et al. 2005).

Materials and Methods

Cell cultures

Human androgen-dependent prostate cancer (LNCaP) and NSCLC (Calu-6) lines, both from American Type Culture Collection (Rockville, MD, USA), were grown at 37 °C in a humidified CO₂ incubator in monolayer. The culture media were RPMI 1640 supplemented with 10 mg/l phenol red, and 10% FBS for LNCaP and MEM supplemented with 10% FBS, 1% non-essential amino acids, 20 mg/dl gentamycin, 200 mM glutamine, and 1 mM sodium pyruvate for Calu-6 cells. Subconfluent cells were collected with 0.05% trypsin/0.02% EDTA (Biochrom, Berlin, Germany) and were seeded into 150 cm² flasks or in 96-well plates depending on the experiments.

Table 1 Human dopamine (D₂R) and somatostatin receptor (SSTR)-binding affinities of the analogues used in this study compared with the SRIF. Bold values represent the highest affinity values of each compound for the respective receptor subtype(s)

<table>
<thead>
<tr>
<th>Ligands</th>
<th>SSTR₁</th>
<th>SSTR₂</th>
<th>SSTR₃</th>
<th>SSTR₄</th>
<th>SSTR₅</th>
<th>D₂R</th>
</tr>
</thead>
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<tr>
<td>SRIF-14</td>
<td>1.9</td>
<td>0.2</td>
<td>1.2</td>
<td>1.7</td>
<td>1.4</td>
<td>–</td>
</tr>
<tr>
<td>BIM-23014</td>
<td>2129</td>
<td>0.75</td>
<td>98</td>
<td>1826</td>
<td>12.7</td>
<td>–</td>
</tr>
<tr>
<td>BIM-23244</td>
<td>1020</td>
<td>0.29</td>
<td>133</td>
<td>1000</td>
<td>0.67</td>
<td>–</td>
</tr>
<tr>
<td>BIM-23120</td>
<td>1000</td>
<td>0.34</td>
<td>412</td>
<td>1000</td>
<td>213.5</td>
<td>–</td>
</tr>
<tr>
<td>BIM-23704</td>
<td>6.25</td>
<td>1.37</td>
<td>43.2</td>
<td>1000</td>
<td>115</td>
<td>–</td>
</tr>
<tr>
<td>BIM-23296</td>
<td>3.6</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>833</td>
<td>788</td>
<td>–</td>
</tr>
<tr>
<td>BIM-53097</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>22.1</td>
</tr>
<tr>
<td>BIM-23206</td>
<td>1152</td>
<td>166</td>
<td>1000</td>
<td>1618</td>
<td>2.4</td>
<td>–</td>
</tr>
<tr>
<td>BIM-23A760</td>
<td>622</td>
<td>0.03</td>
<td>160</td>
<td>1000</td>
<td>42</td>
<td>15</td>
</tr>
</tbody>
</table>

For RNA studies, cells were washed with cold PBS, collected, snap-frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Non-functional pituitary adenoma, obtained from surgery, was taken as positive controls. Total cellular RNA was extracted with the phenol–chloroform method using the Tri Reagent solution (Sigma–Aldrich). Reverse transcription (RT)–PCR analysis for the expression of human D₂R mRNA were as follows: forward (5’-gGCG gAGA CCC CAC TAC AA-3’) and reverse (5’-gAg ggG gAC gAg ACg ACg C-3’).

RNA extraction and reverse transcription-PCR analysis

Products

The SRIF analogues lanreotide (BIM-23014), the bi-specific sstr₂/sstr₅-preferential compound BIM-23244, the sstr₂-preferential compound BIM-23120, the sstr₅-preferential compound BIM-23206, the sstr₁-preferential compound BIM-23926, the bi-specific sstr₁/sstr₅-preferential compound BIM-23704 with a relative lower affinity for sstr₃, the sstr₂/sstr₅/D₂R chimeric compound BIM-23A760, and the D₂R-preferential compound BIM-53097 were used in this study and their respective affinities (Kᵢ) are listed in Table 1. All experimental compounds were kindly provided by IPSEN/Biomeasure (Milford, MA, USA).
Membrane protein extraction

For membrane protein extraction, 5 × 10^6 cells from both the cell lines were seeded into 150 cm² flasks in the appropriate culture media. After 24 h of starvation in serum-free media, the culture medium was replaced by adding the experimental medium containing somatostatin analogues (concentration 10⁻⁷ M). After a 24 h incubation, LNCaP and Calu-6 were solubilized in lysis buffer (20 mM HEPES, 5 mM EDTA, 3 mM EGTA, 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 50 μg/ml bacitracin) and subsequently centrifuged at 20,000 g. Membrane pellet was resuspended with lysis buffer combined with 4 mg/ml dodecyl-D-maltoside, incubated for 1 h on ice, and centrifuged at 20,000 g at 4 °C. Glycosylated proteins were immobilized by recycling the solubilized membrane proteins of the supernatant through a 0.5 ml wheat germ agglutinin (Vector Laboratories, Burlingame, CA, USA) column overnight at 4 °C. The column was eluted with lysis buffer containing 3 mM N,N,N',N'-triacetylchitotriose (Sigma–Aldrich). The protein content of the eluted protein was assessed by Bio-Rad Protein assay (Bio-Rad).

Immunoblot analysis

For D3R, str1, and str5 protein detection and for dimerization studies, 100 μg of LNCaP and Calu-6 membrane receptors and the supernatants obtained after immunoprecipitation were fractioned on 12.5% SDS–PAGE and were electrophoretically transferred to Hybond-C extra nitrocellulose membrane (GE Healthcare, Chalfont, St Giles, UK). Non-specific binding sites were blocked by treating the membranes with Tris-buffered saline–Tween (TBS–T: 0.02 M Tris, 0.137 M NaCl, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 50 μg/ml bacitracin) and subsequently centrifuged at 20,000 g at 4 °C. Membrane pellet was resuspended with lysis buffer combined with 4 mg/ml dodecyl-D-maltoside, incubated for 1 h on ice, and centrifuged at 20,000 g at 4 °C. Glycosylated proteins were immobilized by recycling the solubilized membrane proteins of the supernatant through a 0.5 ml wheat germ agglutinin (Vector Laboratories, Burlingame, CA, USA) column overnight at 4 °C. The column was eluted with lysis buffer containing 3 mM N,N,N',N'-triacetylchitotriose (Sigma–Aldrich). The protein content of the eluted protein was assessed by Bio-Rad Protein assay (Bio-Rad).

Immunoprecipitation

LNCaP and Calu-6 membrane receptors (300 μg) were immunoprecipitated with a mouse anti-human sstr2 (Novus Biologicals), a rabbit anti-human sstr3 polyclonal antibody, and a mouse anti-human D3R monoclonal antibody (Santa Cruz Biotechnology) in the presence of 20 μl protein–A agarose (Sigma–Aldrich) overnight at 4 °C on a rotating shaker. After washing with lysis buffer, the protein–A-conjugated immunocomplex was boiled for 5 min with sample buffer (6% SDS, 0.24 M Tris–HCl 0.5 M, pH 6.8, 30% glycerol, 0.3 mg/ml BPP, 50 mM dithiothreitol) and centrifuged at 10,000 g for 30 s to denature and separate the immunocomplex from protein–A agarose. Supernatants were subjected to immunoblot analysis as described in the previous section.

Cell proliferation studies

To estimate proliferating cells in the S phase of the cell cycle, 15 × 10^3 cells from both the cell lines were seeded into 96-well plates in a final volume of 200 μl/well and incubated at 37 °C in a humidified 5% CO2 atmosphere for 48 h. Then, the culture medium was replaced by adding 180 μl of the experimental medium (RPMI 1640/2% FCS for LNCaP cells and DMEM serum-free 0.1% BSA for Calu-6) containing SRIF analogues and the chimeric compound (10⁻⁹ M). After 48 h (LNCaP) and 24 h (Calu-6), proliferation was measured by cell counting and by measuring thymidine incorporation after the addition of 1 μCi [methyl-3H]-thymidine (3H-Thy; Amersham) during the last 6 h of incubation. 3H-Thy incorporation was determined after collecting cells on glass fibers filter and counting on a scintillation β-counter. Results were obtained by determining the mean value of at least four experiments in eight replicates and expressed as Δ% of the control.

Statistical analysis

Data were expressed as mean ± S.D. for cell proliferation and mean ± S.E.M. for co-immunoprecipitation studies. All data were analyzed by ANOVA to determine the overall differences between the groups, followed by t-test (P < 0.05 was considered significant).

The correlation study was performed by using a linear regression analysis and by calculating the determination coefficient (R²). To minimize variation among different experiments, the results were expressed as relative variation from untreated control value (Δ% of control value).
Results

**LNCaP cell line**

**D2R gene and protein expression** We have already reported the SSTR and D2R profile in Calu-6, as well as the SSTR content in LNCaP (Ferone et al. 2005, Ruscica et al. 2010), hence, before any test we sought to determine the gene and protein expression of D2R in LNCaP cells, by means of RT-PCR and western blot analysis. By using specific oligonucleotides, we detected a product of 523 bp corresponding to the D2R mRNA. Ribosomal 18S was used as internal control for RT-PCR. Moreover, the analysis by immunoblotting of LNCaP cell extracts, using a specific human D2R antibody, showed an immunoreactive band of apparent molecular mass of 40.5–42.5 kDa, depending on protein glycosylation (Fig. 1).

**Receptor interaction** A weak signal of constitutive receptor dimers was demonstrated on the membranes of LNCaP cell line. In particular, we detected the presence of sstr1/sstr2, sstr2/sstr3, sstr3/D2R, and sstr2/D2R complexes (Fig. 2A–D). Treatment with BIM-23704, an sstr1/sstr2-preferential compound (with a relative lower affinity for sstr3 as well), increased the amount of sstr1 co-immunoprecipitated with sstr2 compared with untreated cells (P<0.05; Fig. 2A). This compound did not affect the amount of sstr2/sstr5 complex (Fig. 2B), however, surprisingly, it was also able to increase either the amount of D2R co-immunoprecipitated with sstr5 (P<0.01; Fig. 2C) or the amount of D2R co-immunoprecipitated with sstr2 (P<0.01; Fig. 2D). Treatment with BIM-23244, a compound with a high affinity for sstr1 and sstr5, significantly increased the amount of sstr2 co-immunoprecipitated with sstr5 (P<0.01; Fig. 2B) and the amount of D2R co-immunoprecipitated with sstr5 (P<0.01; Fig. 2C), without affecting the amount of sstr1/sstr2 and the D2R/sstr5 complexes (Fig. 2A and D). As expected, treatment with BIM-23014, a compound with a high affinity for sstr2 and a relative low affinity for sstr5, increased the amount of sstr2 co-immunoprecipitated with sstr5 (P<0.05; Fig. 2B), but it was also able to increase sstr1/sstr2 (P<0.01; Fig. 2A) and D2R/sstr5 complexes (P<0.01; Fig. 2C). Conversely, this compound did not affect the D2R/sstr5 complex (Fig. 2D). BIM-23A760, the chimeric sstr2/D2R compound (with a relative high affinity for sstr5), was one of the most powerful molecules in increasing the amount of D2R co-immunoprecipitated with sstr5 (P<0.01; Fig. 2C) and the amount of D2R co-immunoprecipitated with sstr2 (P<0.01; Fig. 2D), apparently without influencing the sstr1/sstr2 and sstr2/sstr5 receptor complexes (Fig. 2A and B). BIM-53097, the D2R analogue, displayed a profile similar to that of BIM-23A760 (Fig. 2A–D).

Twenty-four hour treatment with all tested substances did not induce any modification of sstr1 expression (Fig. 2E). Similarly, in line with the results obtained by Froidevaux et al. (1999), either the amount of sstr1 or the amount of D2R on cell membrane did not show any difference between untreated and treated cells (data not shown).

**Cell proliferation** The compounds tested in this study produced different antiproliferative effects, as displayed in Fig. 3. According to the above described ability in inducing SSTR/D2R interaction, the chimeric compound BIM-23A760 showed the highest antiproliferative effect (42.30±2.62% versus control) compared with the other test molecules. In line with the potential involvement of sstr1 and sstr5 in the pathophysiology of prostate cancer, BIM-23704 (sstr1/sstr2/sstr3-preferential compound) and BIM-23926 (sstr1 monospecific compound) showed a significant antiproliferative effect (−30.98±6.59 and −36.10±5.58% versus control respectively). The truly bi-specific sstr2/sstr5 compound BIM-23244 and the clinically available SRIF analogue lanreotide (BIM-23014) showed a higher antiproliferative effect (−41.36±4.85 and −33.14±4.60% versus control respectively) as compared with the monospecific sstr2 agonist BIM-23120 alone (−10.89±5.03% versus control) or in combination with the monospecific sstr5 agonist BIM-23206 (−24.18±4.03% versus control). Treatment with BIM-23206 alone showed the lowest antiproliferative effect (−9.60±3.53% versus control), whereas the D2R-preferential compound, BIM-53097, showed a significantly higher antiproliferative effect (−34.4±7.85% versus control), confirming a crucial involvement of D2R activation in the inhibition of cell growth.

Figure 1 RT-PCR and western blot analysis of D2R expression in LNCaP cells: (A) total RNA extracts from LNCaP cells and non-functional pituitary adenoma (NFPa) samples, which served as positive control for D2R, were subjected to RT-PCR analysis using oligonucleotides specific for D2R and 18S ribosomal protein (internal control); (B) cell extracts were subjected to western blot analysis using a D2R antibody. Again tissue extract from NFPa was included as positive control. The bands corresponding to 40.5–42.5 kDa represent two different glycosylated forms of D2R.


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Similarly to LNCaP, baseline constitutive receptor interactions were demonstrated on the membranes of Calu-6 cell line as well. In particular, we detected a significant amount of sstr2 co-immunoprecipitated with sstr5 and sstr5 with D2R on the membranes of these cells (Fig. 4A and B). Treatment with BIM-23014 (a high affinity for sstr2 and a low affinity for sstr5) strongly increased the amount of sstr2 co-immunoprecipitated with sstr5 ($P < 0.01$; Fig. 4A) without affecting sstr5/D$_2$R and sstr2/D$_2$R interactions (Fig. 4B and C). The bi-specific compound with a high affinity for both sstr2 and sstr5, BIM-23244, increased the amount of sstr2 co-immunoprecipitated with sstr5 ($P < 0.01$; Fig. 4A), as well as sstr5 co-immunoprecipitated with D$_2$R ($P < 0.05$; Fig. 4C). Conversely, BIM-23A760, the chimeric compound, with a high affinity for sstr2 and D$_2$R, and a moderate affinity for sstr5, significantly increased the amount of both sstr2 co-immunoprecipitated with sstr5 and sstr5 co-immunoprecipitated with D$_2$R ($P < 0.01$; Fig. 4A and B) without affecting sstr2 co-immunoprecipitated with D$_2$R (Fig. 2C). BIM-53097, the D$_2$R-preferential compound, significantly increased the amount of sstr2 co-immunoprecipitated with sstr5 and sstr5 co-immunoprecipitated with D$_2$R ($P < 0.01$; Fig. 4D), as well as sstr2 co-immunoprecipitated with D$_2$R ($P < 0.05$; Fig. 4C). Twenty-four hours treatment with all the tested substances did not induce any modification of sstr5 expression (Fig. 4D), as well as sstr2 and D$_2$R (data not shown), as already described (Froidevaux et al. 1999).

**Cell proliferation** The chimeric compound BIM-23A760 resulted significantly more effective in inhibiting cell proliferation compared with all the other compounds, tested. For example, BIM-23A760 strongly inhibited cell proliferation compared with all the other compounds tested.

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**Figure 2** Co-immunoprecipitation studies: glycosylated membrane proteins (300 μg) obtained after treatment of LNCaP with the SRIF analogues and the chimeric compound (10$^{-9}$ M) were analyzed for the presence of receptor dimers. On the top of each panel the histogram represents the densitometric analysis of the corresponding band calculated as mean ± S.E.M. of three different experiments. On the bottom of each panel a representative immunoblot is shown. (A) Immunoprecipitation of membrane receptors with a mouse anti-human sstr$_2$ antibody and immunoblotting with a rabbit anti-human sstr1 antibody (in order to avoid the interference of γ-globulin with the band corresponding to the sstr1). The band corresponding to 60 kDa represents the amount of sstr1 co-immunoprecipitated with sstr2. (B) Immunoprecipitation of membrane receptors with a rabbit anti-human sstr5 antibody and immunoblotting with a mouse anti-human sstr2 antibody. The band corresponding to 45 kDa represents the amount of sstr2 co-immunoprecipitated with sstr5. (C) Immunoprecipitation of membrane receptors with a rabbit anti-human sstr5 antibody and immunoblotting with a mouse anti-human D$_2$R antibody. The band corresponding to 40.5 kDa represents the amount of D$_2$R co-immunoprecipitated with sstr5. (D) Immunoprecipitation of membrane receptors with a rabbit anti-human sstr5 antibody and immunoblotting with a mouse anti-human D$_2$R antibody. The band corresponding to 40.5 kDa represents the amount of D$_2$R co-immunoprecipitated with sstr2. (E) A representative sstr1 immunoblot showing unmodified bands of this receptor after a 24 h treatment. On the top of the panel a tubulin immunoblot is shown as internal control. WB, western blot; IP, immunoprecipitation; CONTR, control (untreated cells). *$P<0.05$; **$P<0.01$. 

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**Calu-6 cell line**

**Receptor interaction** Similarly to LNCaP, baseline constitutive receptor interactions were demonstrated on the membranes of Calu-6 cell line as well. In particular, we detected a significant amount of sstr2 co-immunoprecipitated with sstr5 and sstr5 with D$_2$R on the membranes of these cells (Fig. 4A and B). Treatment with BIM-23014 (a high affinity for sstr2 and a low affinity for sstr5) strongly increased the amount of sstr2 co-immunoprecipitated with sstr5 ($P < 0.01$; Fig. 4A) without affecting sstr5/D$_2$R and sstr2/D$_2$R interactions (Fig. 4B and C). The bi-specific compound with a high affinity for both sstr2 and sstr5, BIM-23244, increased the amount of sstr2 co-immunoprecipitated with sstr5 ($P < 0.01$; Fig. 4A), as well as sstr5 co-immunoprecipitated with D$_2$R ($P < 0.05$; Fig. 4C). Conversely, BIM-23A760, the chimeric compound, with a high affinity for sstr2 and D$_2$R, and a moderate affinity for sstr5, significantly increased the amount of both sstr2 co-immunoprecipitated with sstr5 and sstr5 co-immunoprecipitated with D$_2$R ($P < 0.01$; Fig. 4A and B) without affecting sstr2 co-immunoprecipitated with D$_2$R (Fig. 2C). BIM-53097, the D$_2$R-preferential compound, significantly increased the amount of sstr5 co-immunoprecipitated with D$_2$R ($P < 0.01$; Fig. 4D), as well as sstr2 co-immunoprecipitated with D$_2$R ($P < 0.05$; Fig. 4C). Twenty-four hours treatment with all the tested substances did not induce any modification of sstr5 expression (Fig. 4D), as well as sstr2 and D$_2$R (data not shown), as already described (Froidevaux et al. 1999).
involvement of D2R in the regulation of cell functions, Ksstr5-preferential compounds, BIM-23120 and BIM-23206, molecules at a concentration of 10⁻⁹ M in the presence of ³H-Thymidine (1 µCi). Results, obtained from eight replicates of four different experiments, are expressed as percent of cell proliferation (inhibition) ± s.d. with respect to control (untreated cells), **P<0.01; ***P<0.001.

alone or in combination (−30.54±3.15% versus control). BIM-23244, the sstr2- and sstr5-preferential compound, resulted as effective as BIM-23014 in inhibiting ³H-Thy incorporation (−15.84±1.13% versus control and −11.64±2.32% versus control respectively). The sstr2- and sstr5-preferential compounds, BIM-23120 and BIM-23206, tested alone, did not show a significant inhibition of cell proliferation (−2.25±1.71 and −3.66±0.08% versus control respectively), even though these molecules displayed an additive effect (−7.00±1.88% versus control) when tested in combination (Fig. 5). Confirming an important involvement of D2R in the regulation of cell functions, BIM-53097 showed also a significant inhibition of cell proliferation (−17.34±1.95% versus control).

Correlations We investigated the correlations between receptor interaction and cell proliferation of the treated cells by comparing the ligand-induced receptor co-immunoprecipitation with the inhibition of cell proliferation (both evaluated as Δ% of control). The amount of sstr2 co-immunoprecipitated with sstr5 was not correlated (R² 0.0003; P=0.5) with the inhibition of cell proliferation in both cell lines. Conversely, the amount of sstr5 co-immunoprecipitated with D2R was directly and significantly correlated (R² 0.87; P=0.001) with the inhibition of cell proliferation in both cell lines (Fig. 6).

Discussion Several studies focusing on the effect of SRIF and DA analogues on the control of cell secretion and proliferation have demonstrated a broad profile of action, both in vivo and in vitro, in different tissues besides the neuroendocrine ones (Lambers et al. 2002, Schally & Nagy 2003). There are also increasing evidences that SRIF and DA receptors can interact on cell membrane forming homo– or heterodimers with an enhanced functional activity (Ferone et al. 2009). Moreover, almost all studies carried out on cell lines showed receptor dimerization in transfected models highly expressing at least two receptor subtypes (Rocheville et al. 2000a, Pfeiffer et al. 2001, Ren et al. 2003). In this study, we have used two tumor cell lines constitutively expressing four (LNCaP) and three (Calu–6) out of the five SRIF receptors (Ferone et al. 2005, Ruscica et al. 2010) and the D2R. These non-neuroendocrine cell lines, because of their constitutive and relatively low receptor expression, represent a useful model to investigate the physiological mechanisms involved in protein membrane interaction, such as receptor dimerization. Since recently new SRIF analogues with a specific affinity for given receptor subtypes, and SRIF/DA chimeric compounds have been

Figure 3 Proliferation studies: 15×10⁵ LNCaP cells were seeded into 96-wells plates and incubated for 48 h with the experimental molecules at a concentration of 10⁻⁹ M in the presence of ³H-Thymidine (1 µCi). Results, obtained from eight replicates of four different experiments, are expressed as percent of cell proliferation (inhibition) ± s.d. with respect to control (untreated cells), **P<0.01; ***P<0.001.

Figure 4 Co-immunoprecipitation studies: glycosylated membrane proteins (300 µg) obtained after treatment of Calu-6 cells with the SRIF and DA analogues and the chimeric compound (10⁻⁹ M) were analyzed for the presence of receptor dimers. On the top of each panel the histogram represents the densitometric analysis of the corresponding band calculated as mean±S.E.M. of three different experiments. On the bottom of each panel a representative immunoblot is shown. (A) Immunoprecipitation of membrane receptors with a mouse anti-human sstr2 antibody and immunoblotting with a rabbit anti-human sstr2 antibody (in order to avoid the interference of γ-globulin with the band corresponding to the sstr2). The band corresponding to 45 kDa represents the amount of sstr2 co-immunoprecipitated with sstr2. (B) Immunoprecipitation of membrane receptors with a mouse anti-human D2R antibody and immunoblotting with a rabbit anti-human sstr2 antibody. The band corresponding to 45 kDa represents the amount of sstr2 co-immunoprecipitated with D2R. (C) Immunoprecipitation of membrane receptors with a rabbit anti-human sstr2 antibody and immunoblotting with a mouse anti-human D2R antibody. The band corresponding to 40.5 kDa represents the amount of D2R co-immunoprecipitated with sstr2. (D) A representative sstr5 immunoblot showing unmodified bands of this receptor after a 24 h treatment. On the top of the panel a tubulin immunoblot is shown as internal control. WB, western blot; IP, immunoprecipitation; CONTR, control (untreated cells). *P<0.05; **P<0.01.
developed (Ferone et al. 2005, Saveau & Jaquet 2009), in this study we have investigated the effect of these compounds on receptor interaction, and correlated the occurrence of co-immunoprecipitated receptors with the impact on cell proliferation. Since we already demonstrated a dose–response effect of these compounds in the inhibition of cell proliferation (Ferone et al. 2005), in this study, we have performed all experiments using the concentration of compounds that demonstrated about a 50% inhibitory effect (10^{-9} M).

According to the baseline expression of SSTR subtypes in the two cell lines (Ferone et al. 2005, Ruscica et al. 2010) and to the ineffectiveness of a 24 h treatment on receptor expression, we tested specific SRIF analogues in order to study the different combinations of (potential) receptor interaction. Indeed, in LNCaP cell line, we tested the bi-specific SRIF compounds targeting sstr1, sstr2, and sstr5, and in Calu-6 cell line, the bi-specific SRIF compounds targeting sstr2 and sstr5.

Since we have demonstrated the expression of D2R in both the cell lines, we also tested the sstr2/sstr5/D2R chimeric compound and the D2R analogue in order to evaluate the involvement of D2R in membrane receptor interaction.

The results of western blot and co-immunoprecipitation experiments clearly demonstrated that all SSTRs investigated were able to co-immunoprecipitate with other SSTRs and/or with D2R. Moreover, the results of this study confirmed the ability of SSTRs and D2R to interact on plasma membrane, even in the absence of ligands. On the basis of a strong specificity of the receptors subtype antibodies we used, we considered the co-immunoprecipitated receptor as an expression of the presence of constitutive heterodimer. Moreover, besides observing receptor co-immunoprecipitation in basal conditions, we found that treatment with SRIF analogues and with an SRIF/DA chimeric compound can differently modulate the amount of the potential dimers on plasma membranes.

As far as LNCaP cell line is concerned, all compounds targeting sstr2 showed the ability to increase the interaction between almost all the receptors studied, in particular the interaction between SSTR and D2R. As a matter of fact, we observed that lanreotide was able to increase the amount of sstr2 co-immunoprecipitated with D2R, as well as BIM-23704, despite its prevalent affinity for sstr2/sstr2/sstr3. As expected, the chimeric compound BIM-23A760 drastically increased the amount of SSTR co-immunoprecipitated with D2R, resulting in one of the most effective antiproliferative agents among all tested molecules. This last finding suggests a crucial role of D2R in the mechanism involved in the control of tumor growth in these cells, as confirmed by the significant antiproliferative effect and by the increase in the amount of sstr2 co-immunoprecipitated with D2R exerted by the monospecific D2R compound, BIM-53097. Since the sstr2 monospecific compound failed to display a significant antiproliferative effect, and sstr1 and sstr5 but not sstr2 are the receptor subtypes mostly involved in the pathophysiology of prostate cancer, we deduced that sstr2 might mainly function as a ‘scaffold’ for other receptors in the formation of heterodimers.

As far as Calu-6 cell line is concerned, we observed that sstr2 and sstr5 receptors exert a role in the inhibition of cell growth only when activated synergistically. This finding is supported by the effect of the sstr2/sstr5 bi-specific compound, BIM-23244, either in the inhibition of cell growth or in the increase in sstr2 co-immunoprecipitated with sstr5. Moreover, Calu-6 cell line displayed a greater sensibility to the chimeric compounds compared with that observed in LNCaP cells, showing an increase in the amount of sstr5 co-immunoprecipitated with D2R. Moreover, even though the chimeric compound BIM-23A760 was basically ineffective in modulating sstr2/D2R co-immunoprecipitation, it displayed a significant antiproliferative effect. However, considering the affinity of BIM-23A760 for sstr3 as well, this apparent discrepancy can be explained by the predominant involvement of this latter receptor subtype and of D2R, instead of sstr2, in this cell line. Indeed, the inhibition

Figure 5 Proliferation studies: 15×10^3 Calu-6 cells were seeded into 96-well plates and incubated for 48 h with the experimental molecules at a concentration of 10^{-9} M in the presence of 3H-Thymidine (1 μCi). Results, obtained from eight replicates of four different experiments, are expressed as percent of cell proliferation (inhibition) ± s.d. with respect to control (untreated cells). *P<0.05; **P<0.01; ***P<0.001.

Figure 6 Correlation between sstr2/sstr5 and D2R/sstr5 interaction and cell proliferation in LNCaP (triangles) and Calu-6 (squares). On the x-axis the arbitrary density units (AU ratio) of calculated density (∆%) and on the y-axis the ∆% of thymidine incorporation versus control are reported. The results were obtained by using a linear regression analysis calculating the determination coefficient (R²).

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of cell proliferation exerted by the sstr2-preferential compound, BIM-23120, was negligible. This finding is in agreement with the increase in sstr5 co-immunoprecipitated with D2R induced by BIM-53097 (D2R-preferential compound), supporting again the important role of D2R in the induction of SSTR/D2R interaction, probably correlated to the inhibition of cell proliferation as well.

These data highlight the concept of SSTRs as receptors with different functions depending on the different cells in which they are expressed, and not only from the different receptor subtype (Ferone et al. 2001, Zatelli et al. 2001, 2005).

In this study, we have found that the amount of SSTRs co-immunoprecipitated with D2R correspond to an enhanced antiproliferative activity. Nevertheless, it remains to be established whether only one receptor of the dimer accounts for the antiproliferative activity, or both receptors (dimer) modulate cell proliferation. Furthermore, we have found that the influence of different dimers on cell proliferation varies in the two different cell types. Dimers with a direct correlation with the antiproliferative effect probably act on the complex cellular system dedicated to the control of cell cycle and apoptosis (Kidd et al. 2008). The evidence that the compounds able to induce receptor dimerization show a strong antiproliferative effect may be partially explained by the cross talk either on the cell membranes, or even at the postmembrane level, of these G-protein-coupled receptor families (Ferone et al. 2009). Indeed, some authors have demonstrated a reduction in receptor internalization rate, due to the ability of one receptor, implicated in the heterodimer, to retain the second receptor on the cell membrane. In this latter phenomenon, an interference with the β-arrestin system is probably involved (Grant et al. 2008). Another possible explanation of the enhanced antiproliferative action of heterodimers (especially SSR/DR dimers) may be the increased D2R activity observed in the cell lines transfected with sstr3 and D2R after the treatment with SRIF (Baragli et al. 2007). Studies focused on signal transmission at the postmembrane level showed that SRIF/DA receptor activation (largely sstr2/D3R) may result in the phosphorylation of c-Jun N-terminal kinase(B) and inhibition of transcription of the cyclin Ki-67, resulting in growth arrest (Kidd et al. 2008).

On the other hand, the presence of enhanced dimers without a direct correlation with cell proliferation sheds light into the complexity of the ‘dimer system’ that shows a peculiar behavior (far from being completely understood) both at the membrane and postmembrane levels.

In conclusion, the results of this study confirm that the activity of SSTRs and DA receptors could be driven by the specific cell types in which they are expressed, and demonstrate that the dimers behave similarly. Therefore, not only the receptor profiles but also the cell types, as well as the dimerization, are responsible for the final effect of a given ligand. Moreover, in the two cell systems evaluated in this study the only dimer positively correlated with an enhanced antiproliferative effect is the sstr5/D2R one. This finding opens new perspectives for the use of the new chimeric compounds, already used in phase 2 studies in neuroendocrine tumors. However, further studies are warranted to characterize the postreceptor mechanisms involved in the enhanced potency following receptor dimer activation.

**Declaration of interest**

MDC is an employer of IPSEN. The other authors have nothing to disclose.

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**References**


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