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 (−30·98%). BIM-23244 (sstr₂-sstr₅ selective agonist) significantly increased the co-immunoprecipitation of sstr₂/sstr₅, and induced a −41·36% 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.

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Introduction

Somatostatin (or somatotropin release-inhibiting factor, SRIF) acts through five specific G-protein-coupled membrane receptors (SSTRs), code named sstr_{1–5}, 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 clinical 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 pathophysiology 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,

Pfeiffer *et al.* 2001, Patel *et al.* 2002, Grant *et al.* 2004a,b, 2008). 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.

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/sstr₂-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 (K_i) are listed in Table 1. All experimental compounds were kindly provided by IPSEN/Biomeasure (Milford, MA, USA).

RNA extraction and reverse transcription-PCR analysis

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 gene coding for D₂R was performed on total RNA samples. The RT reaction was carried out in a 20 μ l volume, using a commercially available kit (Superscript VILO cDNA Synthesis kit; Invitrogen). The primers used to detect the expression of human D₂R mRNA were as follows: forward (5'-gCg gAC AgA CCC CAC TAC AA-3') and reverse (5'-AAg ggC ACg Tag AAg gAg AC-3').

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)

Ligands	sstr ₁	sstr ₂	sstr ₃	sstr ₄	sstr ₅	D ₂ R
SRIF-14	1.9	0.2	1.2	1.7	1.4	–
BIM-23014	2129	0.75	98	1826	12.7	–
BIM-23244	1020	0.29	133	1000	0.67	–
BIM-23120	1000	0.34	412	1000	213.5	–
BIM-23704	6.25	1.37	43.2	1000	115	–
BIM-23296	3.6	> 1000	> 1000	833	788	–
BIM-53097	–	–	–	–	–	22.1
BIM-23206	1152	166	1000	1618	2.4	–
BIM-23A760	622	0.03	160	1000	42	15

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^{-9} 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- β -malto-side, 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*-triacetylchitotriose (Sigma-Aldrich). The protein content of the eluted protein was assessed by Bio-Rad Protein assay (Bio-Rad).

Immunoblot analysis

For D₂R, sstr₁, and sstr₅ 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, and 0.5% Tween 20; pH 7.6 with 1 M HCl) containing 5% non-fat dried milk for 1 h at 22 °C on a rotating shaker. After three washes with TBS-T, membranes were incubated for 16 h at 4 °C with a 1:500 dilution of mouse anti-human D₂R monoclonal antibody, rabbit anti-human sstr₁ polyclonal antibody, rabbit anti-human sstr₂ polyclonal antibody, rabbit anti-human sstr₅ polyclonal antibody (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 1:500 dilution of a mouse anti-human sstr₂ polyclonal antibody (Novus Biologicals, Littleton, CO, USA) according to the experiment, in TBS-T containing 3% BSA. Membranes were washed three times with TBS-T and then incubated for 1 h at 22 °C with 1:2000 dilution of HRP-linked anti-rabbit/mouse IgG (according to the primary antibody), washed as before, and immersed for 0.5–1 min in the chemiluminescence-detection solution. Subsequently, membranes were exposed for 0.5 min to generate immunoblots. As internal control, membranes used for sstr₁ and sstr₅ expressions were stripped and reprobbed with an antitubulin monoclonal antibody (1:2000; 1A2 clone MoAb, Sigma).

Quantification of the digitized bands was performed in three different experiments (*n* = 3) by UN-SCAN-IT

software (Silk Scientific, Orem, UT, USA) according to the software instructions. In brief, for each band, the net optical density level was automatically determined by subtracting the background optical level from the total raw optical density level and the mean was calculated. Data were expressed as arbitrary density units and were normalized according to the total amount of each receptor subtype after a 24 h treatment.

Immunoprecipitation

LNCaP and Calu-6 membrane receptors (300 µg) were immunoprecipitated with a mouse anti-human sstr₂ (Novus Biologicals), a rabbit anti-human sstr₅ polyclonal antibody, and a mouse anti-human D₂R 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 BPB, 50 mM dithiothreitol) and centrifuged at 1000 *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 (*R*²). 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 oligoprimers, 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/sstr₅, sstr₅/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 sstr₂ 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/sstr₂ 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/sstr₂ 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 sstr₂ 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 ($42.30 \pm 2.62\%$ versus control) compared with the other test molecules. In line with the potential involvement of sstr₁ and sstr₃ 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 $-36.10 \pm 5.58\%$ 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 $-33.14 \pm 4.60\%$ versus control respectively) as compared with the monospecific sstr₂ agonist BIM-23120 alone ($-10.89 \pm 5.03\%$ versus control) or in combination with the monospecific sstr₅ agonist BIM-23206 ($-24.18 \pm 4.03\%$ versus control). Treatment with BIM-23206 alone showed the lowest antiproliferative effect ($-9.60 \pm 3.53\%$ versus control), whereas the D₂R-preferential compound, BIM-53097, showed a significantly higher antiproliferative effect ($-34.4 \pm 7.85\%$ 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 oligoprimers 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.

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 $\pm$ 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 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 D₂R antibody. The band corresponding to 40.5 kDa represents the amount of D₂R co-immunoprecipitated with sstr₅. (D) 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. (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). * $P < 0.05$; ** $P < 0.01$.

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 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/D₂R and sstr₂/D₂R 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 sstr₂ co-immunoprecipitated with D₂R ($P < 0.05$; Fig. 4C). Conversely, BIM-23A760, the chimeric compound, with a high affinity

for sstr₂ and D₂R, and a moderate affinity for sstr₅, significantly increased the amount of both sstr₂ co-immunoprecipitated with sstr₅ and sstr₅ co-immunoprecipitated with D₂R ($P < 0.01$; Fig. 4A and B) without affecting sstr₂ co-immunoprecipitated with D₂R (Fig. 2C). BIM-53097, the D₂R-preferential compound, significantly increased the amount of sstr₅ co-immunoprecipitated with D₂R ($P < 0.01$; Fig. 4B), as well as sstr₂ co-immunoprecipitated with D₂R ($P < 0.05$; 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 D₂R (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

Figure 3 Proliferation studies: 15×10^3 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) $\pm$ s.d. with respect to control (untreated cells). ** $P < 0.01$; *** $P < 0.001$.

alone or in combination ($-30.54 \pm 3.15\%$ versus control). BIM-23244, the sstr_2 - and sstr_5 -preferential compound, resulted as effective as BIM-23014 in inhibiting ^3H -Thy incorporation ($-15.84 \pm 1.13\%$ versus control and $-11.64 \pm 2.32\%$ versus control respectively). The sstr_2 - and sstr_5 -preferential compounds, BIM-23120 and BIM-23206, tested alone, did not show a significant inhibition of cell proliferation (-2.25 ± 1.71 and $-3.66 \pm 0.08\%$ versus control respectively), even though these molecules displayed an additive effect ($-7.00 \pm 1.88\%$ versus control) when tested in combination (Fig. 5). Confirming an important involvement of D_2R in the regulation of cell functions, BIM-53097 showed also a significant inhibition of cell proliferation ($-17.34 \pm 1.95\%$ 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 $\Delta\%$ of control). The amount of sstr_2 co-immunoprecipitated with sstr_5 was not correlated (R^2 0.0003; $P = 0.5$) with the inhibition of cell proliferation in both cell lines. Conversely, the amount of sstr_5 co-immunoprecipitated with D_2R was directly and significantly correlated (R^2 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 (Lamberts *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 D_2R . 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 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 $\pm$ 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 sstr_5 antibody (in order to avoid the interference of γ -globulin with the band corresponding to the sstr_5). The band corresponding to 45 kDa represents the amount of sstr_5 co-immunoprecipitated with sstr_2 . (B) Immunoprecipitation of membrane receptors with a mouse anti-human D_2R antibody and immunoblotting with a rabbit anti-human sstr_5 antibody. The band corresponding to 45 kDa represents the amount of sstr_5 co-immunoprecipitated with D_2R . (C) Immunoprecipitation of membrane receptors with a rabbit anti-human sstr_2 antibody and immunoblotting with a mouse anti-human D_2R antibody. The band corresponding to 40.5 kDa represents the amount of D_2R co-immunoprecipitated with sstr_2 . (D) A representative sstr_5 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). * $P < 0.05$; ** $P < 0.01$.

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) $\pm$ s.d. with respect to control (untreated cells). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

developed (Ferone *et al.* 2005, Saveanu & 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 sstr_1 , sstr_2 , and sstr_5 , and in Calu-6 cell line, the bi-specific SRIF compounds targeting sstr_2 and sstr_5 .

Since we have demonstrated the expression of D_2R in both the cell lines, we also tested the $\text{sstr}_2/\text{sstr}_5/\text{D}_2\text{R}$ chimeric compound and the D_2R analogue in order to evaluate the involvement of D_2R 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 D_2R . Moreover, the results of this study confirmed the ability of SSTRs and D_2R 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 sstr_2 showed the ability to increase the interaction between almost all the receptors studied, in particular the interaction between SSTR and D_2R . As a matter of fact, we observed that lanreotide was able to increase the amount of sstr_5 co-immunoprecipitated with D_2R , as well as BIM-23704, despite its prevalent affinity for $\text{sstr}_1/\text{sstr}_2/\text{sstr}_3$. As expected, the chimeric compound BIM-23A760 drastically increased the amount of SSTR co-immunoprecipitated with D_2R , resulting in one of the most effective antiproliferative agents among all tested molecules. This last finding suggests a crucial role of D_2R 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 sstr_2 co-immunoprecipitated with D_2R exerted by the monospecific D_2R compound, BIM-53097. Since the sstr_2 monospecific compound failed to display a significant antiproliferative effect, and sstr_1 and sstr_3 , but not sstr_2 , are the receptor subtypes mostly involved in the pathophysiology of prostate cancer, we deduced that sstr_2 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 sstr_2 and sstr_5 receptors exert a role in the inhibition of cell growth only when activated synergistically. This finding is supported by the effect of the $\text{sstr}_2/\text{sstr}_5$ bi-specific compound, BIM-23244, either in the inhibition of cell growth or in the increase in sstr_2 co-immunoprecipitated with sstr_5 . 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 sstr_5 co-immunoprecipitated with D_2R . Moreover, even though the chimeric compound BIM-23A760 was basically ineffective in modulating $\text{sstr}_2/\text{D}_2\text{R}$ co-immunoprecipitation, it displayed a significant antiproliferative effect. However, considering the affinity of BIM-23A760 for sstr_5 as well, this apparent discrepancy can be explained by the predominant involvement of this latter receptor subtype and of D_2R , instead of sstr_2 , in this cell line. Indeed, the inhibition

Figure 6 Correlation between $\text{sstr}_2/\text{sstr}_5$ and $\text{D}_2\text{R}/\text{sstr}_5$ interaction and cell proliferation in LNCaP (triangles) and Calu-6 (squares). On the x-axis the arbitrary density units (AU ratio) of calculated density ($\Delta\%$) and on the y-axis the $\Delta\%$ of thymidine incorporation versus control are reported. The results were obtained by using a linear regression analysis calculating the determination coefficient (R^2).

of cell proliferation exerted by the *sstr*₂-preferential compound, BIM-23120, was negligible. This finding is in agreement with the increase in *sstr*₅ co-immunoprecipitated with D₂R induced by BIM-53097 (D₂R-preferential compound), supporting again the important role of D₂R in the induction of SS2R/D₂R interaction, probably correlated to the inhibition of cell proliferation as well.

These data highlight the concept of SS2Rs 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 SS2Rs 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 SS2R/DR dimers) may be the increased D₂R activity observed in the cell lines transfected with *sstr*₂ 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 *sstr*₂/D₂R) 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 SS2Rs 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 *sstr*₅/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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