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**REGULATION OF PROSTATE CANCER CELL PROLIFERATION BY
SOMATOSTATIN RECEPTOR ACTIVATION**

ABBREVIATED TITLE: SRIF receptor activation in prostate cancer

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ABSTRACT

Although some evidence supports the antitumoral effects of somatostatin (SRIF) and related agonists, the available data in prostate cancer (PCa) model systems and clinical studies are few, conflicting and not conclusive. This study investigated the effects of lanreotide and new mono- and bi-specific SRIF agonists on proliferation, ligand-driven SRIF receptor (sst) dimerization and secretory pattern of the IGF system in LNCaP cells, a model of androgen-dependent PCa. LNCaP expressed all sst_s, but sst₄. Among them, sst₁ and sst₃ were inversely regulated by serum concentration. Sst₁/sst₂ and sst₂/sst₅ dimers were constitutively present and further stabilized by treatment with BIM-23704 (sst₁/sst₂) and BIM-23244 (sst₂/sst₅), respectively. Dose-response studies showed that lanreotide and BIM-23244 were significantly more potent in inhibiting LNCaP cell proliferation than BIM-23120 (sst₂) and BIM-23206 (sst₅) alone or in combination. Treatment with BIM-23296 (sst₁) markedly reduced cell proliferation, whereas exposure to BIM-23704 resulted in a lower cell growth inhibition. The antiproliferative effects of BIM-23244, lanreotide and BIM-23704 were unchanged, reduced and abolished by the sst₂ antagonist BIM-23627, respectively. All SRIF analogs caused a significant induction in p27^{Kip1} and p21 and down-regulation of protein expression of cyclin E, as well as reduced IGF-I and IGF-II secretion. In particular, the administration of exogenous IGF-I, at variance to IGF-II, counteracted the inhibitory effect on cell proliferation of these compounds. Moreover, SRIF agonists reduced endogenous IGFBP-3 proteolysis. These results show that, in LNCaP cells, activation of sst₁ and sst₂/sst₅ results in relevant antiproliferative/antisecretive actions.

INTRODUCTION

Somatostatin (SRIF) is an inhibitory tetradecapeptide hormone with exocrine, endocrine, paracrine, and autocrine activities, which plays an important regulatory role in several cell functions, including inhibition of endocrine secretion and cell proliferation (Hejna et al., 2002; Tejeda et al., 2006). Most of the effects of SRIF and of its currently available analogs are mediated via five different G protein-coupled receptor (GPCRs), codenamed sst₁₋₅ (Patel 1999). Sst_s are expressed in a tissue- and subtype-selective manner in both normal and neoplastic cells, and the majority of SRIF target tissues express multiple sst_s (Reubi et al., 2001). Recent data suggest that when sst_s are coexpressed may interact forming homo- and hetero-dimers also with other GPCRs, altering their original pharmacological and functional profiles (Krantic et al., 2004).

Prostate cancer (PCa) is the most frequently diagnosed malignant neoplasm among men in the Western world and remains the second leading cause of cancer-related deaths, with an incidence estimated at 12% in the European Union and at 29% in the USA (Djavan 2007). In addition to the traditional anti-androgen hormonal therapy, commonly used in androgen-dependent PCa, some evidence suggest SRIF receptors as a target for imaging and treatment of PCa cancer with SRIF analogs (Kalkner et al., 1998; Nilsson et al., 1995; Thakur et al., 1997). Indeed, sst_s have been identified in the smooth muscle nodules of both normal and hyperplastic prostatic tissues (Reubi et al., 1995), in human PCa peritumoral veins (Reubi 2003) and in specimens of primary human PCa with Gleason score ranging from 6 to 10 (Halmos et al., 2000).

Few data exist about the selected antiproliferative effect of SRIF and related agonists on PCa model systems. In particular, SRIF was shown to reduce the proliferation of LNCaP cells (Brevini et al., 1993) and adjuvant therapy with lanreotide (sst₂ and partial sst₅ agonist) was found effective in slowing cancer growth in castrated Copenhagen rats bearing Dunning R-3327-H prostate tumors (Bogden et al., 1990). Regarding clinical studies, the results obtained in trials conducted on advanced hormone-refractory prostate cancer patients using lanreotide are conflicting and not conclusive (Figg et al., 1995; Maulard et al., 1995) and thus the ideal

SRIF analog still needs to be determined. Moreover, lanreotide and octreotide (ssr_2/ssr_5 agonist) have been shown to potentiate the antitumoral effect of docetaxel *in vitro* (Erten et al., 2009; Lo Nigro et al., 2008). In addition, the SRIF analog vapreotide has been conjugated to doxorubicin and then used as a carrier molecule to inhibit PC-3 cell proliferation (Plonowski et al., 1999).

SRIF effect on tumors may be the result of direct antiproliferative action and indirect suppression of the secretion of growth-promoting hormones and growth factors, involved in the control of tumor cell growth. In particular, increased IGF-I serum levels seem associated with increased risk of PCa (Djavan et al., 2001; Russell et al., 1998); in some experimental models, progression to androgen independence has been linked to an increased expression of IGF-I and IGF-IR (Nickerson et al., 2001).

Among the different available *in vitro* PCa models, the epithelial LNCaP cells, a line established from supraclavicular lymph node metastases (Horoszewicz et al., 1980), represent a useful experimental tool, since they maintain several characteristics of human PCa, such as the dependence on androgen, the presence of androgen receptors, and the capability to produce a series of androgen-regulated prostate-secreted proteins, such as prostatic specific antigen (PSA) and acid phosphatase (Horoszewicz et al., 1983). The aim of the present study was to investigate the effects of the activation of ssr_s by using new mono- and bi-specific SRIF agonists on: a) LNCaP cell proliferation, including the evaluation of proteins involved in the regulation of the cell cycle; b) ssr_s dimerization; and c) the secretory pattern of IGF-I/-II.

Materials and methods

Cell Cultures. LNCaP and Calu-6, a human non-small cell lung cancer cells (NSCLS), used as a positive control for ssr protein expression, both from American Type Culture Collection (Rockville, MD), were grown at 37°C in a humidified CO₂ incubator in monolayer. The culture medium for LNCaP was RPMI 1640, with 10 mg/L phenol red (Biochrom, Berlin, Germany), and 10% FBS (Gibco, Grand Island, NY); for Calu-6 cells, the medium was MEM supplemented with 10% FBS, 1% non essential amino acids, 20 mg/dL gentamycin, 200 mM glutamine, and 1 mM sodium pyruvate. Subconfluent cells were harvested with 0.05% trypsin/0.02% EDTA (Biochrom, Berlin, Germany) and were seeded in Petri dishes (Becton-Dickinson, Plymouth, UK) or in 96 well-plates (Viewplate-96; Perkin Elmer, Milan, Italy) depending on the experiments. Notably, according to our previous studies (Ruscica et al., 2006), the experimental protocol for proliferation studies included a switch of FBS concentration from 10% to 2%, which allowed to maintain the cultures in good conditions.

RNA Extraction and RT-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 (NFPA) and benign prostatic hyperplasia, obtained from surgery, were taken as positive controls (for ssr_1 , ssr_{2A} , ssr_3 , ssr_5) and for ssr_4 , respectively). Total cellular RNA was extracted with the phenol-chloroform method using the Tri Reagent solution (Sigma-Aldrich, Milan, Italy). RT-PCR analysis of the expression of the genes coding for SRIF and ssr_s was performed on total RNA samples, quantified after an initial DNAase digestion step using the Deoxyribonuclease I kit (Sigma-Aldrich, Milan, Italy). The RT reaction was carried out in a 20 ml volume, using a commercially available kit (Superscript VILO[®] cDNA Synthesis Kit; Invitrogen, Milan, Italy). Negative control reactions omitting reverse transcriptase were carried out in parallel, confirming that no sample contamination by genomic DNA occurred (not shown). The primers used to detect the expression of the genes coding for human SRIF, human ssr_s (ssr_1 , ssr_{2A} , ssr_3 , ssr_4 and ssr_5) and 18 s ribosomal protein were as follows: SRIF forward (5'- gAT gCT gTC CTg CCg CCT CCA g -3') and SRIF reverse (5'- ACA ggA TgT gAA AgT CTT CCA -3'); ssr_1 forward (5'- ATg gTg gCC CTC AAg gCC gg -3') and ssr_1 reverse (5'- CgC ggT ggC gTA

ATA gTC AA -3'); sst_{2A} forward (5'- gCC AAg ATg AAg ACC ATC AC -3') and sst_{2A} reverse (5'- gAT gAA CCC TgT gTA CCA AgC -3'); sst₃ forward (5'- CgT CAg Tgg CgT TCT gAT CC-3') and sst₃ reverse (5'- TgT gCC gCA ggA CCA CAT A -3'); sst₄ forward (5'- CAT ggT CgC TAT CCA gTg CAT-3') and sst₄ reverse (5'- gAA ggA TCA CgA AgA TgA CCA-3'); sst₅ forward (5'- CCg TCT TCA TCA TCT ACA Cgg -3') and sst₅ reverse (5'- ggC CAg gTT gAC gAT gTT gA -3'); 18 s forward (5'- CTC gCT CCT CTC CTA CTT gg -3') and 18 s reverse (5'- CCA TCg AAA gTT gAT Agg gC -3'). Liquid controls and reactions without RT resulted in negative amplifications. The identity of the PCR products was confirmed by DNA sequencing (not shown).

RNA analysis of sst_s by real-time PCR. Since, as mentioned above, FBS supplementation of culture medium in cell proliferation studies switched from standard conditions (10%) to 2%, we assessed whether such variation affected sst_s gene and protein expression. To this aim, real-time RT-PCR was carried out using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Milan, Italy). The ribosomal protein L13a (RPL13A) was used as internal control: RPL13A forward (5' GCGGCTGCCGAAGATG 3'), RPL13A reverse (5' GGCCTCGACCATCAAGCA 3') and RPL13A probe (5' CAGGACCTGCACCTCC 3'). Each sample in triplicate underwent real-time PCR analysis for sst₁, sst₃ and RPL13A. All probes used (custom TaqMan MGB probes, Applied Biosystems, Milan, Italy) contained a reporter fluorescent dye 6-carboxy-fluorescein (FAM) at the 5' end, and a minor-groove-binder moiety and a non-fluorescent quencher dye at the 3' end. Briefly, the 25 µL reaction mixture consisted of 12.5 µL 2x TaqMan Master Mix (Applied Biosystems, Milan, Italy), 1.25 µL of specific 20X gene expression Assay Mix, 1 µL cDNA and 10.25 µL nuclease-free water. Calculation of the relative expression levels of the target mRNAs was conducted based on the relative standard curve method (singleplex).

Immunoblotting. Membrane receptors were obtained from LNCaP and Calu-6 cells by solubilization in lysis buffer (20 mM HEPES, 5 mM EDTA, 3 mM EGTA, 150 mM NaCl, 1 mM PMSF, 10 µg/mL soybean trypsin inhibitor, 10 µg/mL leupeptin, 50 µg/mL bacitracin). and subsequent centrifugation at 20,000 x g. Membrane pellet was resuspended with lysis buffer additioned with 4 mg/mL dodecyl-D-maltoside, incubated 1 h on ice and centrifugated at 20.000 x 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) column overnight at 4°C. The column was eluted with lysis buffer containing 3 mM N,N',N'-triacetylchitotriose (Sigma-Aldrich, St. Louis, MO). The protein content of the eluted protein was assessed by Bradford assay. Glycosylated proteins (10 µg) was denatured and fractionated on 12.5% SDS-PAGE and then transferred electrophoretically to Hybond C-extra nitrocellulose membranes. After transfer, nonspecific binding sites were blocked by treating membranes with Tris-buffered saline-Tween (TBS-T) containing 5% nonfat dried milk for 1 h at 22°C. To evaluate the presence of SRIF receptors, after five washes with TBS-T, membranes were incubated for 16 h at 4°C with a 1:500 dilution of sst₁, sst_{2A}, sst₃, sst₄ and sst₅ (Santa Cruz Biotechnology, CA) polyclonal antibodies and incubated for 1 h at 22°C with 1:2,000 dilution of horseradish peroxidase-linked anti-rabbit IgG. To evaluate the cell cycle arrest, membranes were then incubated with a diluted solution of the primary antibody (anti-p21, 1:100; anti-p27^{Kip1}, 1:100; anti-cycline E, 1:100 (Cell Signaling Technology, MA); anti-tubulin, 1:2,000 (Sigma-Aldrich, St. Louis, MO) and the subsequent incubation with a secondary antibody (1:8,000; 1:5,000; 1:5,000 and 1:8,000, respectively) conjugated with peroxidase was performed at room temperature for 2 h. Membranes were washed with TBS-T, as before, immersed in the chemiluminescence detection solution and exposed to radiographic film.

Immunoprecipitation (IP). Membrane protein preparations (300 µg) were subjected to immunoprecipitation with anti-sst₂ and -sst₅ polyclonal antibody (Santa Cruz Biotechnology,

CA) in the presence of 20 μ L of protein A-Agarose (Santa Cruz Biotechnology, CA). After washing steps 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 bromophenol blue, 50 mM DTT) to denature and separate the immunocomplex from protein A-Agarose. After a centrifugation at 1000xg for 30 sec, the supernatants were processed as previously described in the “immunoblotting” section.

Sst_s-selective agonists. The SRIF analogs: lanreotide, bi-specific sst₂/sst₅-preferential compound BIM-23244 (Gordon et al., 2003), sst₂-preferential compound BIM-23120 (degli Uberti et al., 2002), sst₅-preferential compound BIM-23206 (Coy et al., 1990), bi-specific sst₁/sst₂-preferential compound BIM-23704, sst₁-preferential compound BIM-23296 and sst₂ antagonist BIM-23627 used in this study and their respective affinities (IC₅₀) to the different sst_s are listed in Table 1. All experimental compounds were kindly provided by Biomeasure (Milford, MA), while SRIF-14 (SRIF) was purchased from Sigma-Aldrich (Milan, Italy).

Cell proliferation studies. To estimate the effect of SRIF analogs alone or in combination with exogenous IGF-I and -II on cell proliferation, 15*10³ LNCaP cells were seeded in a 96-well plates in a final volume of 200 μ L per well and incubated at 37°C in a humidified 5% CO₂ atmosphere for 48 h. The culture medium was replaced by adding 180 μ L of experimental medium (RPMI 1640/2% FBS) containing human SRIF and SRIF analogs (range: 10⁻⁷-10⁻¹¹ M), when used alone or at the concentration of 10⁻⁹ M, when used in combination with IGF-I and IGF-II (both at 7 nM). Thus, the incubation was carried on for further 48 h. Treatments were renewed every 24 h. Twenty microliter of 100 μ M 5'-bromo-2'-deoxyuridine (BrdU labeling solution, Delfia cell proliferation kit, Perkin Elmer, Milan, Italy) were added to the cultures 24 h before the end of the incubation. Cells were subsequently fixed (fix solution, 100 μ L per well) for 30 min and then incubated, at room temperature, for further 120 min with a primary monoclonal antibody against BrdU (0.5 μ g/mL) conjugated with Europium (Eu; Anti-BrdU-Eu working solution). Finally, after adding a specific inducer solution (200 μ L/well), the Eu-fluorescence was measured in a time-resolved fluorometer. To exclude any unspecific binding of both BrdU and anti-BrdU-Eu, for every experiment a row without cells (only medium) has been analyzed. Results (Eu-counts) were obtained by determining the mean value of at least 4 experiments in 8 replicates.

Conditioned medium. To prepare conditioned media (CM), LNCaP cells were plated in 100-mm Petri dishes (Becton-Dickinson, Plymouth, UK) and grown in RPMI (Biochrom, Berlin, Germany) with 10% FBS, at 37°C in a humidified CO₂ incubator in monolayer, until they reached 60–70% confluence. Thus, cells were washed twice with PBS with Ca⁺⁺/Mg⁺⁺, and cultured for 36 h in RPMI supplemented with 0.1% BSA (Sigma-Aldrich, Milan, Italy) in the presence or absence of SRIF and SRIF analogs at the concentration of 10⁻⁹ M (which represents the dose to which all SRIF analogs show an bi-specific sst₁/sst₂-preferential compound effect) and that of sst₂ antagonist at the concentration of 10⁻⁷ M (Zatelli et al., 2001). Treatments with all compounds did not modify the survival rate of the cells (viable cells > 95% according to trypan blue exclusion) or induce any morphological change (data not shown). The supernatants were collected, centrifuged at 1,000xg and stored at -80°C until testing.

Chromatographic profile of the secreted IGF-I and IGF-II. CM was acidified by 18 h dialysis at 4°C against 1 M acetic acid in Spectra-Por 6 membranes (molecular weight (mw) cut off, 1000 Da), lyophilized and resuspended with 0.1 M acetic acid-0.15 M NaCl (pH of the mixture < 3). Then, IGF-I and IGF-II were separated from IGFBPs by gel filtration on FPLC Superdex-75 column equilibrated with 0.1 M acetic acid-0.15 M NaCl, pH 2.75. Fractions were pooled at 0.1 Kav intervals, lyophilized, reconstituted in 1 mL PBS and analyzed for

IGF-I and for IGF-II. IGF-I and IGF-II contents of media were evaluated in the fractions eluted from the Superdex-75 column in the mw range of the free peptide.

Measurement of IGF-I and IGF-II in the conditioned medium. IGF-I was measured by using a commercially available radioimmunoassay (RIA) kit (Biosource; Nivelles, Belgium). The sensitivity of the RIA assay was 0.02 ng/mL; the intra-assay and inter-assay coefficients of variation were 6.0% and 7.5%, respectively. IGF-II was measured by an immunoradiometric assay (IRMA) (Diagnostic Systems Laboratories, Texas). The sensitivity of the IRMA assay was 0.13 ng/mL; the intra-assay and inter-assay coefficients of variation were 5.3% and 8.7%, respectively. IGF-I and -II concentrations in the conditioned media were normalized to the protein content.

rh-IGFBP-3 proteolysis. To evaluate the effect of SRIF analog treatment on the protease activity of CM on rh-IGFBP-3, aliquots of CM corresponding to 5×10^5 cells were lyophilized, reconstituted with 100 μ L of distilled H₂O and incubated with 50 ng of deglycosylated rh-IGFBP-3 in the presence of 2 mM CaCl₂ at 37°C for 5 h. The amount of residual intact rh-IGFBP-3 was visualized by immunoblotting, by using a 1:5000 dilution of anti-IGFBP-3 antiserum leading to the identification of a single band of 30 kDa (Tressel et al., 1991). Deglycosylated rh-IGFBP-3 and anti-IGFBP-3 antiserum were provided by Dr. A. Sommer (INSMED Inc., Richmond, VA).

Analysis of the data. Statistical analysis was performed using the Prism statistical analysis package (GraphPad Software, San Diego, CA). Data are given as Mean $\pm$ SD of three independent experiments. Differences between treatment groups were evaluated by one way ANOVA and were considered significant at $P \leq 0.05$.

RESULTS

Sst₅ gene and protein expression. Before any test with SRIF agonists, we sought to determine the gene and protein expression pattern of sst₁₋₅ in LNCaP cells, by means of RT-PCR and Western blot analysis. By using specific oligoprimers, we detected specific products of 318 bp for sst₁, 414 bp for sst_{2A}, 95 bp for sst₃ and 226 bp for sst₅. Ribosomal 18 s was used as internal control for RT-PCR (250 bp) (Fig. 1A). Moreover, the analysis of LNCaP cell extracts by immunoblotting using specific human sst₅ antibodies, showed immunoreactive bands of apparent mw of 65 kDa (sst₁), 45-90 kDa (sst_{2A}), 45-70 kDa (sst₃), 40-70 kDa (sst₅), depending on protein glycosylation (Fig. 1B). Neither gene nor protein expression of sst₄ was detected (Fig. 1A, B).

Experimental conditions modulate sst₁ and sst₃ gene and protein expression. The experimental conditions for LNCaP cell proliferation studies include a switch of FBS supplement from 10% (regular culture conditions) to 2%, according to our previous experience (Ruscica et al. 2006). The presence of 2% FBS was associated with up-regulation of sst₁ and sst₃ expression, while no changes were observed for sst_{2A} and sst₅ (not shown). In particular, real-time RT-PCR analysis showed that sst₁ mRNA and sst₃ mRNA expression were up-regulated (mean increment at 48 h, ~2.4-fold and ~2.9-fold, respectively) (Fig. 2A). Similarly, protein expression of the sst₁ and sst₃ were increased (for both ~ +200%) (Fig. 2B).

Cell proliferation studies. LNCaP cells were subjected to a 48-h dose-response study (10^{-11} to 10^{-7} M) with SRIF and different SRIF agonists. All these agents were found to inhibit cell proliferation to some extent with a peculiar profile related to the sst specificity of each compound. The native ligand, SRIF, was significantly effective in inhibiting cell growth, over the whole dose range tested, (-43/-57%; $P < 0.01$) (Fig. 3A). The bi-specific sst₂/sst₅-preferential agonist BIM-23244 was able to reduce LNCaP cell growth by 30% at 10^{-11} M,

then reaching a plateau of -40% at 10^{-10} - 10^{-7} M ($p < 0.01$). Lanreotide resulted very effective at the lower concentrations (-49/-46% at 10^{-11} - 10^{-10} M), with a lesser but still significant activity at 10^{-9} - 10^{-7} M (-27/-23%) (Fig. 3A). The mono-specific ss_{t2} preferential compound BIM-23120 showed a maximal cell growth inhibition at 10^{-11} M (-21%, $p < 0.01$), and progressively showed a lower efficacy with increasing doses (Fig. 3B). The mono-specific ss_{t5} preferential compound BIM-23206 showed a statistically significant inhibitory effect only at 10^{-11} M (-15%, $p < 0.01$) (Fig. 3B). To evaluate the possible effect played by the simultaneous activation of ss_{t2} and ss_{t5} receptors, we treated LNCaP cells with BIM-23120 (ss_{t2}) and BIM-23206 (ss_{t5}) in combination. Interestingly, this treatment resulted in an additive effect, compared to each compound alone, over the 10^{-11} - 10^{-8} M range, however reaching a lesser inhibition (-19/-27, $P < 0.01$) compared to BIM-23244 (ss_{t2}/ss_{t5}) and lanreotide (Fig. 3B). Treatment with the ss_{t1} -preferential agonist BIM-23296 markedly reduced cell proliferation (-28/-36% at 10^{-11} - 10^{-7} M; $P < 0.01$), whereas exposure to the bi-specific ss_{t1}/ss_{t2} -preferential agonist BIM-23704 resulted in a significant, but lower cell growth inhibition over the whole range of doses (-23/-13% at 10^{-11} - 10^{-7} M; $P < 0.01$), with maximal efficacy at 10^{-8} M (Fig. 3C). In order to clarify the importance of ss_{t2} recruitment in the induction of the antiproliferative activity of the bi-specific compounds, BIM-23704 (ss_{t1}/ss_{t2}), BIM-23244 (ss_{t2}/ss_{t5}) and lanreotide, cells were treated with the selective ss_{t2} antagonist BIM-23627 (10^{-7} M), alone or in combination with these agonists. BIM-23627 alone did not alter LNCaP cell growth compared with untreated control cells (Fig. 3D). In the presence of the antagonist, the effect of lanreotide was dramatically reduced from -49/-23% to -11/-4% (at 10^{-11} - 10^{-7} M) (Fig. 3D), whereas the effect of BIM-23244 was not modified (data not shown). Moreover, the inhibition of BrdU incorporation induced by BIM-23704 was totally abolished, in the presence of the antagonist, over the 10^{-11} - 10^{-8} M dose range (Fig. 3E). The above-reported effects on cell proliferation with BrdU were paralleled and confirmed by the [3 H]thymidine incorporation assay (data not shown). Thus, ss_{t1} - and ss_{t2}/ss_{t5} -preferential SRIF agonists were found to be the most effective inhibitors of LNCaP growth.

Sst₁/sst₂ and sst₂/sst₅ dimerization study. To assess whether treatment with bi-specific SRIF analogs may modulate sst heterodimerization, IP/WB experiments were performed. A small amount of ss_{t1}/ss_{t2} and ss_{t2}/ss_{t5} heterodimers was constitutively present on LNCaP cell membranes (Fig. 4A/B). Treatment with BIM-23704 (ss_{t1}/ss_{t2}) increased the amount of ss_{t1} co-immunoprecipitated with ss_{t2} , which may probably reflect a stabilization of the ss_{t1}/ss_{t2} heterodimers (Fig. 4A). Treatment with lanreotide increased the amount of the ss_{t2} receptor co-immunoprecipitated with the ss_{t5} , also probably reflecting the stabilization of ss_{t2}/ss_{t5} heterodimers. Treatment with the ss_{t2}/ss_{t5} -preferential compound BIM-23244 resulted in an even more pronounced effect compared to lanreotide (Fig. 4B). Compared with the constitutive levels, BIM-23244 did not affect the amount of ss_{t1}/ss_{t2} heterodimers (Fig. 4A) and BIM-23704 did not affect that of ss_{t2}/ss_{t5} heterodimers (Fig. 4B). Thus, the bi-specific ss_{t1}/ss_{t2} and ss_{t2}/ss_{t5} -preferential compounds enhanced the stabilization of the corresponding heterodimers.

Effect of SRIF analogs treatment on protein expression of p27^{Kip1}, p21 and cyclin E in LNCaP cells. The effect of SRIF analogs on the expression of proteins involved in cell cycle arrest was then evaluated. Relative density data revealed that all SRIF analogs caused a significant induction in p27^{Kip1} and p21 and down-regulation of protein expression of cyclin E, compared to cells treated with media alone (fig. 5). In particular, BIM-23244 (ss_{t2}/ss_{t5}) caused 1.6- and 2.4-fold increases of the protein expression of p27^{Kip1} and p21, respectively. as well as a significant decrement (-34%) in cyclin E level. BIM-23926 (ss_{t1}) was able to increase p27^{Kip1} and p21 (1.74 and 2.63 fold. respectively) and reduce (-11%) cyclin E protein levels.

SRIF agonists down-regulate IGF-I and IGF-II secretion in LNCaP cells. The IGF system is

known to contribute to PCa progression through systemic and paracrine/autocrine mechanisms. Specifically, LNCaP cells were shown to secrete small amounts of IGFs. Thus, we evaluated the ability of SRIF agonists to regulate IGF-I and -II secretion by these cells, after a 48-h treatment. BIM-23244 ($ss_{2/5}$) and lanreotide significantly inhibited IGF-I (-91% and -36%, respectively) and IGF-II (-59 and -68%, respectively) secretion. The inhibitory effects of BIM-23244 on both IGFs and lanreotide on IGF-I were abolished by co-treatment with the $ss_{2/5}$ antagonist BIM-23627. Conversely, treatment with BIM-23704 ($ss_{1/2}$) produced a +33% increase of IGF-I and a -43% decrease of IGF-II secretion (Table 2), which were dramatically reduced in the presence of BIM-23627. Among the monospecific ligands, BIM-23926 ($ss_{1/2}$) markedly reduced IGF-I (-85%) and IGF-II (-68%) secretion, BIM-23120 ($ss_{2/5}$) significantly inhibited IGF-I (-79%) and slightly stimulated (+19%) IGF-II secretion, BIM-23206 ($ss_{5/6}$) inhibited both IGF-I (-49%) and IGF-II (-86%) secretion (Table 2). In conclusion, the secretion of IGFs by LNCaP cells appears to be markedly affected by almost all SRIF analogs tested.

Effect IGFs on SRIF agonists-induced inhibition of LNCaP cell proliferation. In order to study the role of IGFs on growth of PCa cells, we evaluated the effect of exogenous IGF-I and IGF-II, tested alone or in combination with SRIF agonists, on LNCaP cell proliferation. As shown in Table 3, a 48-h treatment with IGF-I alone significantly increased LNCaP cell proliferation (+18%). Moreover, the inhibitory effects of lanreotide, BIM-23244, BIM-23120, BIM-23206 and BIM-23926 was fully counteracted by the addition of IGF-I. On the contrary, the administration of exogenous IGF-II did not change LNCaP cell proliferation, nor affected the antiproliferative action of each SRIF agonist.

SRIF agonists reduce the proteolytic activity of LNCaP conditioned media. Another IGF system component with relevance to the local control of PCa progression is IGFBP-3, which binds IGF-I and also undergoes to degradation by proteolysis, resulting in lower IGF-I binding. Thus, we evaluated the ability of CM from LNCaP cells to cleave exogenous rh-IGFBP-3 by WB. After a 5-h incubation with CM from untreated LNCaP cells, intact rh-IGFBP-3 was dramatically reduced, compared to vehicle (PBS). Such extensive proteolytic cleavage was partially reduced by treatment of LNCaP cells with SRIF analogs. Moreover, combined treatment with the antagonist BIM-23627 and the mono-specific or bi-specific $ss_{5/6}$ analogs abolished the antiproteolytic effect induced by SRIF analogs (Fig. 4C). SRIF analogs, therefore, seem able to interfere with the IGF system also by reducing the proteolytic degradation of IGFBP-3.

DISCUSSION

The present study reports the substantial evidence for an important role of the SRIF system in controlling PCa cell proliferation. Specifically, four ($ss_{1-2,3-5}$) out of five $ss_{5/6}$ receptors were found to be expressed in the LNCaP cell line, an *in vitro* model of human androgen-dependent PCa. Their activation by selective SRIF agonists resulted in a significant antiproliferative effect with a peculiar pattern according to receptor subtype, ligand affinity and, possibly, receptor dimerization. Moreover, such treatments were also able to modulate the profile of the IGF system, known to be involved in PCa progression (Russell et al., 1998).

The $ss_{5/6}$ expression profile observed in LNCaP cells partially differs from that reported in other studies in the same cell line, which showed either the expression of all receptors (Liu et al., 2008) or of some of them (Dizeyi et al., 2002; Taylor et al., 1994; Zapata et al., 2002). Moreover, a great heterogeneity exists in the pattern of $ss_{5/6}$ expression in other PCa cell lines and normal prostate and PCa tissue (Halmos et al., 2000; Sinisi et al., 1997). Interestingly, $ss_{1/2}$ and $ss_{3/4}$ were markedly up-regulated by different experimental conditions, an observation in line with previous evidence of $ss_{5/6}$ modulation by hormones. Indeed, it has been recently shown that in PCa cells, treatment with 5-aza decitabine and trichostatin in

combination increased ssr_5 mRNA content (Liu et al. 2008), similarly to the effects observed in other cell systems after treatment with glucocorticoids or with estrogens (Djordjijevic et al., 1998; Kimura et al., 2008; Viguerie et al., 1987; Visser-Wisselaar et al., 1996). It has been also documented that hormone therapies may be responsible for a change in ssr_s expression in PCa (Reubi 2004). Thus, one may speculate that combined androgen blockade therapy in PCa patients might possibly up-regulate the expression of some ssr_s , thereby improving some diagnostic applications (*i.e.*, *in vivo* scintigraphy by using radiolabelled-SRIF analogs) (Khan et al., 2008), as well as the therapeutic efficacy of SRIF analogs.

Although few data exist about the selected antiproliferative effect of SRIF and related agonists on PCa model systems (Bogden et al. 1990; Brevini et al., 1993), these studies did not fully address the potential and selective role of the different ssr_s in PCa progression. Thus, we evaluated the effects of selective SRIF agonists on LNCaP cell growth. BIM-23244 (the ssr_2/ssr_5 preferential compound) and lanreotide (a ssr_2 preferential compound with a moderate affinity for ssr_5) were significantly more potent in inhibiting LNCaP cell proliferation than the mono-specific ssr_2 - and ssr_5 -preferring analogs (BIM-23120 and BIM-23206, respectively), tested either alone or in combination. These differences between the mono- and the bi-specific compounds might be due to the ability of BIM-23244 and, to a lesser extent, of lanreotide to stabilize or enhance ssr_2/ssr_5 dimerization (corresponding to the amount of ssr_2 co-immunoprecipitated with ssr_5), as suggested by the increased stabilization (and/or number) of the ssr_2/ssr_5 dimers on the cell membrane after treatment with these compounds (Fig. 4B). This finding is in agreement with the ability of ssr_5 in regulating ssr_2 trafficking (Sharif et al., 2007) and/or its retain within the membrane (Ben-Shlomo et al., 2005), and might also explain the efficacy of BIM-23244 over a wider dose range compared with lanreotide. Moreover, in contrast with the reversibility of the effect observed with lanreotide, the increased activity of BIM-23244 was not counteracted by the ssr_2 antagonist, BIM-23267. Furthermore, in LNCaP cells, BIM-23244, in agreement with previous observations in other cell systems, such as human pituitary adenoma and NSCLC Calu-6 cells (Ferone et al., 2005; Saveanu et al., 2001) closely mimicked the effects of native SRIF. Differently from human medullary thyroid TT cells, where BIM-23206 completely counteracts the suppressive activity of BIM-23120 (Zatelli et al., 2001), in LNCaP cells the effects of these agonists were both directed toward a significant antiproliferative effect, suggesting not only tissue-specific activity, but also a tissue-specific interactions between these two ssr_s . The different cross-talk between ssr_s in different districts may partially explain the functional tissue-specificity of an ubiquitous hormone such as SRIF. Dose-response experiments showed that the ssr_1 -preferential compound BIM-23296 negatively affected cell proliferation, with a greater potency than ssr_2 and ssr_5 agonist, possibly due to ssr_1 up-regulation and/or to a low agonist-induced internalization of ssr_1 (Hofland et al., 2003; Nouel et al., 1997; Sarret et al., 1999). Notably, ssr_1 was found to be the most abundantly expressed SRIF receptor in PCa tissue (Halmos et al., 2000; Reubi et al., 1995). The results obtained with the ssr_1/ssr_2 -preferential agonist BIM-23704 suggest that the simultaneous recruitment of these two receptors, although able to stabilize ssr_1/ssr_2 dimerization, is less effective in terms of antiproliferation and fully sensitive to specific ssr_2 antagonism, than ssr_2/ssr_5 activation.

Moreover, our data suggest that the antiproliferative effects of these SRIF agonists on LNCaP cells, may, at least in part, result from cell cycle arrest in the G_0/G_1 phase, as suggested by the observed up-regulation of the cyclin-dependent kinase inhibitors p27 and p21 and the inhibition of cyclin E. Along with these direct actions on cell cycle, SRIF may also regulate tumor growth by indirect action suppressing the secretion of growth-promoting hormones and growth factors (Lamberts et al., 2002). In addition to systemic suppression of the GH-IGF-I axis, SRIF analogs may also affect paracrine and autocrine IGF-I loops within the tumoral prostatic tissue (Pietrzkowski et al., 1993). LNCaP cells have been shown to secrete low amounts of IGF-I and IGF-II and to express some IGF-binding proteins (IGFBPs), with prevalence of IGFBP-3 (Kawada et al., 2006). Interestingly, we found that

1 treatment with SRIF agonists markedly affected the secretory profile of the IGF system,
 2 consequently inhibiting cell growth.

3 Our study also demonstrated the ability of exogenous IGF-I, at variance to IGF-II, to
 4 counteract the inhibitory effect on cell proliferation of all SRIF analogs tested. This is in
 5 agreement with the finding of a mitogenic effect of IGF-I alone on LNCaP cells, which is not
 6 evident after incubation with IGF-II. These findings could be explained by the different
 7 relative amounts of IGFBP-3 (which has higher binding affinity for IGF-I with respect to
 8 IGF-II) and IGFBP-2 (which has higher binding affinity for IGF-II with respect to IGF-I)
 9 present in LNCaP conditioned medium (data not shown). In fact, since IGFBP-3 is almost
 10 totally proteolysed by PSA secreted by LNCaP cells, the free form of IGF-I may increase,
 11 leading to an enhanced IGF-I bioactivity. On the contrary, IGFBP-2, which is present in high
 12 concentration, leads to a prevalent inhibition of IGF-II effect.

13 Indirect confirmation of this hypothesis comes from the clinical experience where a positive
 14 correlation between IGF-I and IGFBP-3 and no correlation between IGF-II and IGFBP-2 and
 15 prostate cancer was observed (Roddam et al., 2008).

16 In particular, IGF-I secretion was dramatically reduced by lanreotide and BIM-23244
 17 ($ss_{2/5}$ agonists), BIM-23926 (ss_1 agonist), BIM-23120 (ss_2 agonist), BIM-23206 (ss_5
 18 agonist), but not BIM-23704 (ss_1/ss_2 agonist). Moreover, ss_2 antagonist BIM-23627
 19 abolished the stimulatory effect of BIM-23704 and the inhibitory effect of lanreotide and
 20 BIM-23244. IGF-II secretion was also markedly reduced by all SRIF agonists except BIM-
 21 23120. Such effects were counteracted by BIM-23627 only with bi-specific compounds,
 22 further emphasizing the need of simultaneous recruitment of both receptors. SRIF agonist-
 23 induced reduction of IGF-I/II levels may impair the activity of the autocrine loop, thereby
 24 contributing to growth inhibition, although in the present study we could not establish the
 25 extent of the specific contribution of reduced/increased IGFs to this phenomenon.

26 Deglycosylated rh-IGFBP-3 underwent an extensive proteolytic cleavage in untreated cells,
 27 possibly due to the activity of PSA, which is secreted by these cells and shows specific
 28 proteolytic activity toward IGFBP-3 (Birnbaum et al., 1994). Interestingly, all compounds
 29 increased the amount of intact rh-IGFBP-3, probably due to a reduced PSA production
 30 (Ruscica et al., unpublished data), whereas treatment with the ss_2 antagonist BIM-23627,
 31 alone or in combination with mono- or bi-specific ss_2 analogs, abolished this effect. SRIF
 32 agonist-induced increase of intact rh-IGFBP-3 might reduce free IGF-I availability resulting
 33 in a further suppression of paracrine and autocrine IGF-I loop.

34 The present study highlights that ss_s receptor modulation may lead to inhibition of
 35 androgen-dependent PCa cell growth through a specific receptor isoform involvement and
 36 dimerization. Among all tested compounds, a particular relevance emerged for ss_1 - and
 37 ss_2/ss_5 -preferring agonists, which, at least in terms of antiproliferative effects, more closely
 38 mimicked the activity profile of SRIF. Thus, these results expand the role of ss_s receptors as
 39 potential therapeutic targets for androgen-dependent PCa. The development of SRIF analogs
 40 with high affinity for each ss_s , as well as of pan-ligands and bi-specific compounds (Boerlin
 41 et al., 2003) together with the novel insight of ss_s pathophysiology (Patel 1999), has helped
 42 to better understand the role of the ss_s depending on different biological activity and on
 43 differential expression in human prostatic tissue (Zatelli et al., 2001). Moreover, this is the
 44 first report that provides strong evidence for an inhibitory role of ss_1 activation on PCa cell
 45 proliferation, suggesting that SRIF agonists with enhanced ss_1 affinity and selectivity may
 46 have great potentiality as pharmacological tools for at least androgen-dependent PCa
 47 treatment. In addition, the antiproliferative effect of ss_1 and ss_5 mono-specific agonists may
 48 be due, at least in part, to the inhibition of IGF-I secretion.

49 In conclusion, our results show that ss_1 up-regulation and ss_1 -driven
 50 antiproliferative/antiseecretive actions represent a set of complementary favorable events in
 51 terms of antiproliferative activity. Moreover, the bi-specific ss_2/ss_5 -preferential compound
 52 BIM-23244-promoted enhancing of ss_2/ss_5 dimer stabilization and thereby potential
 53 regulation of ss_2 trafficking, and modulation of IGF secretion, resulting in a marked

1 antiproliferative effect. Conversely, activation of ssr_1/ssr_2 appears however less effective than
2 that of either ssr_1 alone or ssr_2/ssr_5 . These observations represent pharmacological events that
3 may deserve further exploitation at the experimental and possibly clinical levels, also in the
4 context of established cancer therapies, like taxanes and other chemotherapeutic agents.

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FIGURE LEGENDS

Fig. 1. RT-PCR and Western blot analysis of *sst*₁₋₅ expression in LNCaP cells. A) Total RNA extracts from LNCaP cells, non-functional pituitary adenoma (NFPA - *sst*_{1-2A-3-5} positive control) and benign prostatic hyperplasia (BPH - *sst*₄ positive control) samples were subjected to RT-PCR analysis using oligoprimers specific for *sst*₁₋₅ and 18 s ribosomal protein (internal control); B) Cell extracts were subjected to Western blot analysis using antisera against *sst*₁, *sst*_{2A}, *sst*₃, *sst*₄ and *sst*₅. The human Calu-6 NSCLC cell line and a sample of benign prostatic hyperplasia (BPH) were included as positive controls.

Fig. 2. Influence of different culture conditions on *sst*₁ and *sst*₃ gene and protein levels of expression in LNCaP cells. LNCaP cells were cultured either with 10% FBS (standard culture conditions) or with 2% FBS (experimental culture conditions) for 48 h. A) Semiquantitative RT-PCR analysis of *sst*₁ and *sst*₃ gene expression. Parallel amplification of 18 s ribosomal protein mRNA served as internal control. B) Real-time RT-PCR analysis of *sst*₁ and *sst*₃ gene expression. Data are presented as mean fold increment $\pm$ SEM, $n = 3$, $**P < 0.01$ vs. 10% FBS. C) Western blot analysis of *sst*₁ and *sst*₃ in protein extracts from LNCaP cells.

Fig. 3. Effects of SRIF analogs and *sst*₂-selective antagonist BIM-23627 on BrdU-incorporation on LNCaP cells. Mean dose-response growth-inhibition curves obtained with LNCaP cells cultured in 96-well plates for 48 h with: A) lanreotide (Lan), bi-specific *sst*₂/*sst*₅-preferential compound BIM-23244 and SRIF; B) *sst*₂-preferential compound BIM-23120, *sst*₅-preferential compound BIM-23206 and the combination of both; C) *sst*₁-preferential compound BIM-23296 and bi-specific *sst*₁/*sst*₂-preferential compound BIM-23704; D) Lan (10^{-7} - 10^{-11} M) with or without BIM-23627 (Ant; 10^{-7} M) and E) BIM-23704 with or without Ant (10^{-7} M). Control wells were treated with vehicle solution. Results are expressed as mean ($\pm$ SE) % growth inhibition vs. control (-), $n = 8$.

Fig. 4. *Sst₁/sst₂, sst₂/sst₅ heterodimerization and effect of SRIF analogs on rh-IGFBP-3 proteolytic activity of LNCaP cell conditioned medium.* To assess the stabilization of sst₁/sst₂- and sst₂/sst₅-driven heterodimerization, membrane preparations from LNCaP cells were, respectively: A) immunoprecipitated with anti-sst₂ antibody and immunoblotted with anti-sst₁ antibody. The band of 60 kDa corresponds to the sst₁ co-immunoprecipitated with sst₂; B) immunoprecipitated with anti-sst₅ antibody and immunoblotted with anti-sst₂ antibody. The band of 45 kDa corresponds to the sst₂ co-immunoprecipitated with sst₅; C) Exogenous rh-IGFBP-3 was incubated for 5 h at 37°C with conditioned media from LNCaP cultured in the presence or absence of different SRIF analogs (10⁻⁹ M) and sst₂-selective antagonist BIM-23627 (10⁻⁷ M). As internal control exogenous rh-IGFBP-3 was incubated only with vehicle (PBS). The amount of residual intact rh-IGFBP-3 was visualized by immunoblotting. Lanreotide and BIM-23627 are indicated as Lan and Ant, respectively.

IP: immunoprecipitation; WB: Western blot

Fig. 5. *Effect of SRIF analogs treatment on protein expression of p27^{Kip1}, p21 and cyclin E in LNCaP cells.* As detailed in the “Materials and Methods” section, the cells were treated for 48 h with SRIF analogs at the concentration of 10⁻⁹ M and vehicle alone (control). Total cell lysates were prepared for immunoblot analysis. The values above the figures represent the relative density of each band normalized to tubulin. The data shown here are from a representative experiment repeated three times with similar results.

**Table 1. SRIF and SRIF analogs: human somatostatin receptor
subtype (sst₁₋₅) specificity (IC₅₀-nM)**

compound	sst₁	sst₂	sst₃	sst₄	sst₅
SRIF	1.95	0.25	1.2	1.77	1.41
Lanreotide	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-23206	1152	166	1000	1618	2.4
BIM-23926	3.6	>1000	1000	833	788
BIM-23704	6.25	1.37	43.2	1000	115
BIM-23627	2757	6.4	44	423	86.5

Table 2. Effect of treatments with SRIF analogs on IGF-I and IGF-II secretion by LNCaP cells

	Lan	Lan + Ant	BIM-23244	BIM-23244 + Ant	BIM-23704	BIM-23704 + Ant	BIM-23120	BIM-23120 + Ant	BIM-23206	BIM-23926
IGF-I	-36.2 ± 2.3	+10.8 ± 5.2	-91.1 ± 0.7	+21.5 ± 2.0	+33.5 ± 2.4	+3.8 ± 2.4	-75.9 ± 1.2	-50.6 ± 1.1	-49.3 ± 1.4	-85 ± 1.7
IGF-II	-68.1 ± 0.9	-81.1 ± 0.6	-59.2 ± 0.4	+8.10 ± 1.3	-43.3 ± 1.1	+12 ± 1.7	+19.2 ± 1.4	+72.9 ± 1.0	-85.8 ± 1.2	-68.5 ± 1.4

Experiments were conducted in triplicate and the results expressed as mean (% variation) ± SD (basal medium content; IGF-I = 25 ng/mL; IGF-II = 38.2 ng/mL)

Lan, lanreotide; **Ant**, BIM-23627; **IGF**, insulin-like growth factor

Table 3. Effect of 48h-treatment with SRIF agonists, IGF-I and IGF-II on LNCaP cell proliferation

C	IGF-I	Lan	Lan + IGF-I	BIM-23244	BIM-23244 + IGF-I	BIM-23120	BIM-23120 + IGF-I	BIM-23206	BIM-23206 + IGF-I	BIM23926	BIM23926 + IGF-I
	18.5 ± 3.5	-23.1 ± 2.9	-4.1 ± 0.9	-20.8 ± 1.8	-0.9 ± 3.1	-12.2 ± 1.8	13.3 ± 8.3	-12 ± 2.0	9.5 ± 6.7	-18.6 ± 3.4	-0.7 ± 1.3

C	IGF-II	Lan	Lan + IGF-II	BIM-23244	BIM-23244 + IGF-II	BIM-23704	BIM-23704 + IGF-II	BIM-23206	BIM-23206 + IGF-II	BIM23926	BIM23926 + IGF-II
	2.4 ± 7.55	-23.1 ± 2.9	-16.6 ± 3.4	-20.8 ± 1.8	-17.5 ± 3.5	-13.1 ± 0.9	-9.5 ± 0.5	-12 ± 2.0	-8.8. ± 1.1	-13.3 ± 1.9	-9.1 ± 2.9

48-h treatment experiments were conducted in duplicate (each n=8) and the results expressed as mean (% variation) ± SEM.

Lan, lanreotide; **IGF-I and -II**, insulin-like growth factor I and II

Figure 1

Fig. 1

B)

Fig. 2

Fig. 3

Fig 4

Figure 5

Fig. 5