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

3
4
5 **ABBREVIATED TITLE:** SRIF receptor activation in prostate cancer

6
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20
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22 proliferation, somatostatin receptor subtypes dimerization, IGFs system

23
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25

1 ABSTRACT

2
3 Although some evidence supports the antitumoral effects of somatostatin (SRIF) and related
4 agonists, the available data in prostate cancer (PCa) model systems and clinical studies are
5 few, conflicting and not conclusive. This study investigated the effects of lanreotide and new
6 mono- and bi-specific SRIF agonists on proliferation, ligand-driven SRIF receptor (sst)
7 dimerization and secretory pattern of the IGF system in LNCaP cells, a model of androgen-
8 dependent PCa. LNCaP expressed all sst_s, but sst₄. Among them, sst₁ and sst₃ were inversely
9 regulated by serum concentration. Sst₁/sst₂ and sst₂/sst₅ dimers were constitutively present
10 and further stabilized by treatment with BIM-23704 (sst₁/sst₂) and BIM-23244 (sst₂/sst₅),
11 respectively. Dose-response studies showed that lanreotide and BIM-23244 were
12 significantly more potent in inhibiting LNCaP cell proliferation than BIM-23120 (sst₂) and
13 BIM-23206 (sst₅) alone or in combination. Treatment with BIM-23296 (sst₁) markedly
14 reduced cell proliferation, whereas exposure to BIM-23704 resulted in a lower cell growth
15 inhibition. The antiproliferative effects of BIM-23244, lanreotide and BIM-23704 were
16 unchanged, reduced and abolished by the sst₂ antagonist BIM-23627, respectively. All SRIF
17 analogs caused a significant induction in p27^{Kip1} and p21 and down-regulation of protein
18 expression of cyclin E, as well as reduced IGF-I and IGF-II secretion. In particular, the
19 administration of exogenous IGF-I, at variance to IGF-II, counteracted the inhibitory effect
20 on cell proliferation of these compounds. Moreover, SRIF agonists reduced endogenous
21 IGFBP-3 proteolysis. These results show that, in LNCaP cells, activation of sst₁ and sst₂/sst₅
22 results in relevant antiproliferative/antisecretive actions.

25 INTRODUCTION

26
27 Somatostatin (SRIF) is an inhibitory tetradecapeptide hormone with exocrine, endocrine,
28 paracrine, and autocrine activities, which plays an important regulatory role in several cell
29 functions, including inhibition of endocrine secretion and cell proliferation (Hejna et al.,
30 2002; Tejada et al., 2006). Most of the effects of SRIF and of its currently available analogs
31 are mediated via five different G protein-coupled receptor (GPCRs), codenamed sst₁₋₅ (Patel
32 1999). Sst_s are expressed in a tissue- and subtype-selective manner in both normal and
33 neoplastic cells, and the majority of SRIF target tissues express multiple sst_s (Reubi et al.,
34 2001). Recent data suggest that when sst_s are coexpressed may interact forming homo- and
35 hetero-dimers also with other GPCRs, altering their original pharmacological and functional
36 profiles (Krantic et al., 2004).

37 Prostate cancer (PCa) is the most frequently diagnosed malignant neoplasm among men in
38 the Western world and remains the second leading cause of cancer-related deaths, with an
39 incidence estimated at 12% in the European Union and at 29% in the USA (Djavan 2007). In
40 addition to the traditional anti-androgen hormonal therapy, commonly used in androgen-
41 dependent PCa, some evidence suggest SRIF receptors as a target for imaging and treatment
42 of PCa cancer with SRIF analogs (Kalkner et al., 1998; Nilsson et al., 1995; Thakur et al.,
43 1997). Indeed, sst_s have been identified in the smooth muscle nodules of both normal and
44 hyperplastic prostatic tissues (Reubi et al., 1995), in human PCa peritumoral veins (Reubi
45 2003) and in specimens of primary human PCa with Gleason score ranging from 6 to 10
46 (Halmos et al., 2000).

47 Few data exist about the selected antiproliferative effect of SRIF and related agonists on PCa
48 model systems. In particular, SRIF was shown to reduce the proliferation of LNCaP cells
49 (Brevini et al., 1993) and adjuvant therapy with lanreotide (sst₂ and partial sst₅ agonist) was
50 found effective in slowing cancer growth in castrated Copenhagen rats bearing Dunning R-
51 3327-H prostate tumors (Bogden et al., 1990). Regarding clinical studies, the results obtained
52 in trials conducted on advanced hormone-refractory prostate cancer patients using lanreotide
53 are conflicting and not conclusive (Figg et al., 1995; Maulard et al., 1995) and thus the ideal

1 SRIF analog still needs to be determined. Moreover, lanreotide and octreotide (sst_2/sst_5
2 agonist) have been shown to potentiate the antitumoral effect of docetaxel *in vitro* (Erten et
3 al., 2009; Lo Nigro et al., 2008). In addition, the SRIF analog vapreotide has been conjugated
4 to doxorubicin and then used as a carrier molecule to inhibit PC-3 cell proliferation
5 (Plonowski et al., 1999).

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

12 Among the different available *in vitro* PCa models, the epithelial LNCaP cells, a line
13 established from supraclavicular lymph node metastases (Horoszewicz et al., 1980),
14 represent a useful experimental tool, since they maintain several characteristics of human
15 PCa, such as the dependence on androgen, the presence of androgen receptors, and the
16 capability to produce a series of androgen-regulated prostate-secreted proteins, such as
17 prostatic specific antigen (PSA) and acid phosphatase (Horoszewicz et al., 1983). The aim of
18 the present study was to investigate the effects of the activation of sst_5 by using new mono-
19 and bi-specific SRIF agonists on: a) LNCaP cell proliferation, including the evaluation of
20 proteins involved in the regulation of the cell cycle; b) sst_5 dimerization; and c) the secretory
21 pattern of IGF-I/-II.

22 **Materials and methods**

23 *Cell Cultures.* LNCaP and Calu-6, a human non-small cell lung cancer cells (NSCLS), used
24 as a positive control for sst protein expression, both from American Type Culture Collection
25 (Rockville, MD), were grown at 37°C in a humidified CO₂ incubator in monolayer. The
26 culture medium for LNCaP was RPMI 1640, with 10 mg/L phenol red (Biochrom, Berlin,
27 Germany), and 10% FBS (Gibco, Grand Island, NY); for Calu-6 cells, the medium was
28 MEM supplemented with 10% FBS, 1% non essential amino acids, 20 mg/dL gentamycin,
29 200 mM glutamine, and 1 mM sodium pyruvate. Subconfluent cells were harvested with
30 0.05% trypsin/0.02% EDTA (Biochrom, Berlin, Germany) and were seeded in Petri dishes
31 (Becton-Dickinson, Plymouth, UK) or in 96 well-plates (Viewplate-96; Perkin Elmer, Milan,
32 Italy) depending on the experiments. Notably, according to our previous studies (Ruscica et
33 al., 2006), the experimental protocol for proliferation studies included a switch of FBS
34 concentration from 10% to 2%, which allowed to maintain the cultures in good conditions.
35

36 *RNA Extraction and RT-PCR analysis.* For RNA studies, cells were washed with cold PBS,
37 collected, snap-frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Non-
38 functional pituitary adenoma (NFPA) and benign prostatic hyperplasia, obtained from
39 surgery, were taken as positive controls (for sst_1 , sst_{2A} , sst_3 , sst_5) and for sst_4 , respectively).
40 Total cellular RNA was extracted with the phenol-chloroform method using the Tri Reagent
41 solution (Sigma-Aldrich, Milan, Italy). RT-PCR analysis of the expression of the genes
42 coding for SRIF and sst_s was performed on total RNA samples, quantified after an initial
43 DNAase digestion step using the Deoxyribonuclease I kit (Sigma-Aldrich, Milan, Italy). The
44 RT reaction was carried out in a 20 ml volume, using a commercially available kit
45 (Superscript VILO[®] cDNA Synthesis Kit; Invitrogen, Milan, Italy). Negative control
46 reactions omitting reverse transcriptase were carried out in parallel, confirming that no
47 sample contamination by genomic DNA occurred (not shown). The primers used to detect
48 the expression of the genes coding for human SRIF, human sst_s (sst_1 , sst_{2A} , sst_3 , sst_4 and sst_5)
49 and 18 s ribosomal protein were as follows: SRIF forward (5'- gAT gCT gTC CTg CCg
50 CCT CCA g -3') and SRIF reverse (5'- ACA ggA TgT gAA AgT CTT CCA -3'); sst_1
51 forward (5'- ATg gTg gCC CTC AAg gCC gg -3') and sst_1 reverse (5'- CgC ggT ggC gTA
52
53

1 ATA gTC AA -3'); sst_{2A} forward (5'- gCC AAg ATg AAg ACC ATC AC -3') and sst_{2A}
 2 reverse (5'- gAT gAA CCC TgT gTA CCA AgC -3'); sst₃ forward (5'- CgT CAg Tgg CgT
 3 TCT gAT CC-3') and sst₃ reverse (5'- TgT gCC gCA ggA CCA CAT A -3'); sst₄ forward
 4 (5'- CAT ggT CgC TAT CCA gTg CAT-3') and sst₄ reverse (5'- gAA ggA TCA CgA AgA
 5 TgA CCA-3'); sst₅ forward (5'- CCg TCT TCA TCA TCT ACA Cgg -3') and sst₅ reverse
 6 (5'- ggC CAg gTT gAC gAT gTT gA -3'); 18 s forward (5'- CTC gCT CCT CTC CTA CTT
 7 gg -3') and 18 s reverse (5'- CCA TCg AAA gTT gAT Agg gC -3'). Liquid controls and
 8 reactions without RT resulted in negative amplifications. The identity of the PCR products
 9 was confirmed by DNA sequencing (not shown).

10

11 *RNA analysis of sst_s by real-time PCR.* Since, as mentioned above, FBS supplementation of
 12 culture medium in cell proliferation studies switched from standard conditions (10%) to 2%,
 13 we assessed whether such variation affected sst_s gene and protein expression. To this aim,
 14 real-time RT-PCR was carried out using an ABI PRISM 7000 sequence detection system
 15 (Applied Biosystems, Milan, Italy). The ribosomal protein L13a (RPL13A) was used as
 16 internal control: RPL13A forward (5' GCGGCTGCCGAAGATG 3'), RPL13A reverse (5'
 17 GGCCTCGACCATCAAGCA 3') and RPL13A probe (5' CAGGACCTGCACCTCC 3').
 18 Each sample in triplicate underwent real-time PCR analysis for sst₁, sst₃ and RPL13A. All
 19 probes used (custom TaqMan MGB probes, Applied Biosystems, Milan, Italy) contained a
 20 reporter fluorescent dye 6-carboxy-fluorescein (FAM) at the 5' end, and a minor-groove-
 21 binder moiety and a non-fluorescent quencher dye at the 3' end. Briefly, the 25 µL reaction
 22 mixture consisted of 12.5 µL 2x TaqMan Master Mix (Applied Biosystems, Milan, Italy),
 23 1.25 µL of specific 20X gene expression Assay Mix, 1 µL cDNA and 10.25 µL nuclease-free
 24 water. Calculation of the relative expression levels of the target mRNAs was conducted
 25 based on the relative standard curve method (singleplex).

26

27 *Immunoblotting.* Membrane receptors were obtained from LNCaP and Calu-6 cells by
 28 solubilization in lysis buffer (20 mM HEPES, 5 mM EDTA, 3 mM EGTA, 150 mM NaCl, 1
 29 mM PMSF, 10 µg/mL soybean trypsin inhibitor, 10 µg/mL leupeptin, 50 µg/mL bacitracin).
 30 and subsequent centrifugation at 20,000 x g. Membrane pellet was resuspended with lysis
 31 buffer additioned with 4 mg/mL dodecyl-D-maltoside, incubated 1 h on ice and centrifugated
 32 at 20.000 x g at 4°C. Glycosylated proteins were immobilized by recycling the solubilized
 33 membrane proteins of the supernatant through a 0.5-mL wheat-germ agglutinin (Vector
 34 Laboratories, Burlingame, CA) column overnight at 4°C. The column was eluted with lysis
 35 buffer containing 3 mM N,N',N-triacetylchitotriose (Sigma-Aldrich, St. Louis, MO). The
 36 protein content of the eluted protein was assessed by Bradford assay. Glycosylated proteins
 37 (10 µg) was denatured and fractionated on 12.5% SDS-PAGE and then transferred
 38 electrophoretically to Hybond C-extra nitrocellulose membranes. After transfer, nonspecific
 39 binding sites were blocked by treating membranes with Tris-buffered saline-Tween (TBS-T)
 40 containing 5% nonfat dried milk for 1 h at 22°C. To evaluate the presence of SRIF receptors,
 41 after five washes with TBS-T, membranes were incubated for 16 h at 4°C with a 1:500
 42 dilution of sst₁, sst_{2A}, sst₃, sst₄ and sst₅ (Santa Cruz Biotechnology, CA) polyclonal antibodies
 43 and incubated for 1 h at 22°C with 1:2,000 dilution of horseradish peroxidase-linked anti-
 44 rabbit IgG. To evaluate the cell cycle arrest, membranes were then incubated with a diluted
 45 solution of the primary antibody (anti-p21, 1:100; anti-p27^{Kip1}, 1:100; anti-cycline E, 1:100
 46 (Cell Signaling Technology, MA); anti-tubulin, 1:2,000 (Sigma-Aldrich, St. Louis, MO) and
 47 the subsequent incubation with a secondary antibody (1:8,000; 1:5,000; 1:5,000 and 1:8,000,
 48 respectively) conjugated with peroxidase was performed at room temperature for 2 h.
 49 Membranes were washed with TBS-T, as before, immersed in the chemiluminescence
 50 detection solution and exposed to radiographic film.

51

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

1 CA) in the presence of 20 μ L of protein A-Agarose (Santa Cruz Biotechnology, CA). After
2 washing steps with lysis buffer, the Protein-A conjugated immunocomplex was boiled for 5
3 min with sample buffer (6% SDS, 0.24 M Tris-HCl 0.5 M pH 6.8, 30% Glycerol, 0.3 mg/ml
4 bromophenol blue, 50 mM DTT) to denature and separate the immunocomplex from protein
5 A-Agarose. After a centrifugation at 1000xg for 30 sec, the supernatants were processed as
6 previously described in the “immunoblotting” section.

7
8 *Sst_s-selective agonists.* The SRIF analogs: lanreotide, bi-specific sst₂/sst₅-preferential
9 compound BIM-23244 (Gordon et al., 2003), sst₂-preferential compound BIM-23120 (degli
10 Uberti et al., 2002), sst₅-preferential compound BIM-23206 (Coy et al., 1990), bi-specific
11 sst₁/sst₂-preferential compound BIM-23704, sst₁-preferential compound BIM-23296 and sst₂
12 antagonist BIM-23627 used in this study and their respective affinities (IC₅₀) to the different
13 sst_s are listed in Table 1. All experimental compounds were kindly provided by Biomeasure
14 (Milford, MA), while SRIF-14 (SRIF) was purchased from Sigma-Aldrich (Milan, Italy).

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

34
35 *Conditioned medium.* To prepare conditioned media (CM), LNCaP cells were plated in 100-
36 mm Petri dishes (Becton-Dickinson, Plymouth, UK) and grown in RPMI (Biochrom, Berlin,
37 Germany) with 10% FBS, at 37°C in a humidified CO₂ incubator in monolayer, until they
38 reached 60–70% confluence. Thus, cells were washed twice with PBS with Ca⁺⁺/Mg⁺⁺, and
39 cultured for 36 h in RPMI supplemented with 0.1% BSA (Sigma-Aldrich, Milan, Italy) in the
40 presence or absence of SRIF and SRIF analogs at the concentration of 10⁻⁹ M (which
41 represents the dose to which all SRIF analogs show an bi-specific sst₁/sst₂-preferential
42 compound effect) and that of sst₂ antagonist at the concentration of 10⁻⁷ M (Zatelli et al.,
43 2001). Treatments with all compounds did not modify the survival rate of the cells (viable
44 cells > 95% according to trypan blue exclusion) or induce any morphological change (data
45 not shown). The supernatants were collected, centrifuged at 1,000xg and stored at -80°C until
46 testing.

47
48 *Chromatographic profile of the secreted IGF-I and IGF-II.* CM was acidified by 18 h dialysis
49 at 4°C against 1 M acetic acid in Spectra-Por 6 membranes (molecular weight (mw) cut off,
50 1000 Da), lyophilized and resuspended with 0.1 M acetic acid-0.15 M NaCl (pH of the
51 mixture < 3). Then, IGF-I and IGF-II were separated from IGF-BPs by gel filtration on FPLC
52 Superdex-75 column equilibrated with 0.1 M acetic acid-0.15 M NaCl, pH 2.75. Fractions
53 were pooled at 0.1 Kav intervals, lyophilized, reconstituted in 1 mL PBS and analyzed for

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

3
4 *Measurement of IGF-I and IGF-II in the conditioned medium.* IGF-I was measured by using
5 a commercially available radioimmunoassay (RIA) kit (Biosource; Nivelles, Belgium). The
6 sensitivity of the RIA assay was 0.02 ng/mL; the intra-assay and inter-assay coefficients of
7 variation were 6.0% and 7.5%, respectively. IGF-II was measured by an immunoradiometric
8 assay (IRMA) (Diagnostic Systems Laboratories, Texas). The sensitivity of the IRMA assay
9 was 0.13 ng/mL; the intra-assay and inter-assay coefficients of variation were 5.3% and
10 8.7%, respectively. IGF-I and -II concentrations in the conditioned media were normalized to
11 the protein content.

12
13 *rh-IGFBP-3 proteolysis.* To evaluate the effect of SRIF analog treatment on the protease
14 activity of CM on rh-IGFBP-3, aliquots of CM corresponding to 5×10^5 cells were
15 lyophilized, reconstituted with 100 μ L of distilled H₂O and incubated with 50 ng of
16 deglycosylated rh-IGFBP-3 in the presence of 2 mM CaCl₂ at 37°C for 5 h. The amount of
17 residual intact rh-IGFBP-3 was visualized by immunoblotting, by using a 1:5000 dilution of
18 anti-IGFBP-3 antiserum leading to the identification of a single band of 30 kDa (Tressel et
19 al., 1991). Deglycosylated rh-IGFBP-3 and anti-IGFBP-3 antiserum were provided by Dr. A.
20 Sommer (INSMED Inc., Richmond, VA).

21
22 *Analysis of the data.* Statistical analysis was performed using the Prism statistical analysis
23 package (GraphPad Software, San Diego, CA). Data are given as Mean \pm SD of three
24 independent experiments. Differences between treatment groups were evaluated by one way
25 ANOVA and were considered significant at $P \leq 0.05$.

26 27 RESULTS

28
29 *Sst₅ gene and protein expression.* Before any test with SRIF agonists, we sought to determine
30 the gene and protein expression pattern of sst₁₋₅ in LNCaP cells, by means of RT-PCR and
31 Western blot analysis. By using specific oligoprimers, we detected specific products of 318
32 bp for sst₁, 414 bp for sst_{2A}, 95 bp for sst₃ and 226 bp for sst₅. Ribosomal 18 s was used as
33 internal control for RT-PCR (250 bp) (Fig. 1A). Moreover, the analysis of LNCaP cell
34 extracts by immunoblotting using specific human sst₅ antibodies, showed immunoreactive
35 bands of apparent mw of 65 kDa (sst₁), 45-90 kDa (sst_{2A}), 45-70 kDa (sst₃), 40-70 kDa (sst₅),
36 depending on protein glycosylation (Fig. 1B). Neither gene nor protein expression of sst₄
37 was detected (Fig. 1A, B).

38
39 *Experimental conditions modulate sst₁ and sst₃ gene and protein expression.* The
40 experimental conditions for LNCaP cell proliferation studies include a switch of FBS
41 supplement from 10% (regular culture conditions) to 2%, according to our previous
42 experience (Ruscica et al. 2006). The presence of 2% FBS was associated with up-regulation
43 of sst₁ and sst₃ expression, while no changes were observed for sst_{2A} and sst₅ (not shown). In
44 particular, real-time RT-PCR analysis showed that sst₁ mRNA and sst₃ mRNA expression
45 were up-regulated (mean increment at 48 h, ~2.4-fold and ~2.9-fold, respectively) (Fig. 2A).
46 Similarly, protein expression of the sst₁ and sst₃ were increased (for both ~ +200%) (Fig. 2B).

47
48 *Cell proliferation studies.* LNCaP cells were subjected to a 48-h dose-response study (10^{-11} /
49 10^{-7} M) with SRIF and different SRIF agonists. All these agents were found to inhibit cell
50 proliferation to some extent with a peculiar profile related to the sst specificity of each
51 compound. The native ligand, SRIF, was significantly effective in inhibiting cell growth,
52 over the whole dose range tested, (-43/-57%; $P < 0.01$) (Fig. 3A). The bi-specific sst₂/sst₅-
53 preferential agonist BIM-23244 was able to reduce LNCaP cell growth by 30% at 10^{-11} M,

1 then reaching a plateau of -40% at 10^{-10} - 10^{-7} M ($p < 0.01$). Lanreotide resulted very effective
 2 at the lower concentrations (-49/-46% at 10^{-11} - 10^{-10} M), with a lesser but still significant
 3 activity at 10^{-9} - 10^{-7} M (-27/-23%) (Fig. 3A). The mono-specific sst_2 preferential compound
 4 BIM-23120 showed a maximal cell growth inhibition at 10^{-11} M (-21%, $p < 0.01$), and
 5 progressively showed a lower efficacy with increasing doses (Fig. 3B). The mono-specific
 6 sst_5 preferential compound BIM-23206 showed a statistically significant inhibitory effect
 7 only at 10^{-11} M (-15%, $p < 0.01$) (Fig. 3B). To evaluate the possible effect played by the
 8 simultaneous activation of sst_2 and sst_5 receptors, we treated LNCaP cells with BIM-23120
 9 (sst_2) and BIM-23206 (sst_5) in combination. Interestingly, this treatment resulted in an
 10 additive effect, compared to each compound alone, over the 10^{-11} - 10^{-8} M range, however
 11 reaching a lesser inhibition (-19/-27, $P < 0.01$) compared to BIM-23244 (sst_2/sst_5) and
 12 lanreotide (Fig. 3B). Treatment with the sst_1 -preferential agonist BIM-23296 markedly
 13 reduced cell proliferation (-28/-36% at 10^{-11} - 10^{-7} M; $P < 0.01$), whereas exposure to the bi-
 14 specific sst_1/sst_2 -preferential agonist BIM-23704 resulted in a significant, but lower cell
 15 growth inhibition over the whole range of doses (-23/-13% at 10^{-11} - 10^{-7} M; $P < 0.01$), with
 16 maximal efficacy at 10^{-8} M (Fig. 3C). In order to clarify the importance of sst_2 recruitment in
 17 the induction of the antiproliferative activity of the bi-specific compounds, BIM-23704
 18 (sst_1/sst_2), BIM-23244 (sst_2/sst_5) and lanreotide, cells were treated with the selective sst_2
 19 antagonist BIM-23627 (10^{-7} M), alone or in combination with these agonists. BIM-23627
 20 alone did not alter LNCaP cell growth compared with untreated control cells (Fig. 3D). In the
 21 presence of the antagonist, the effect of lanreotide was dramatically reduced from -49/-23%
 22 to -11/-4% (at 10^{-11} - 10^{-7} M) (Fig 3D), whereas the effect of BIM-23244 was not modified
 23 (data not shown). Moreover, the inhibition of BrdU incorporation induced by BIM-23704
 24 was totally abolished, in the presence of the antagonist, over the 10^{-11} - 10^{-8} M dose range (Fig
 25 3E). The above-reported effects on cell proliferation with BrdU were paralleled and
 26 confirmed by the [3 H]thymidine incorporation assay (data not shown). Thus, sst_1 - and
 27 sst_2/sst_5 -preferential SRIF agonists were found to be the most effective inhibitors of LNCaP
 28 growth.

29
 30 *Sst₁/sst₂ and sst₂/sst₅ dimerization study.* To assess whether treatment with bi-specific SRIF
 31 analogs may modulate sst heterodimerization, IP/WB experiments were performed. A small
 32 amount of sst_1/sst_2 and sst_2/sst_5 heterodimers was constitutively present on LNCaP cell
 33 membranes (Fig. 4A/B). Treatment with BIM-23704 (sst_1/sst_2) increased the amount of sst_1
 34 co-immunoprecipitated with sst_2 , which may probably reflect a stabilization of the sst_1/sst_2
 35 heterodimers (Fig. 4A). Treatment with lanreotide increased the amount of the sst_2 receptor
 36 co-immunoprecipitated with the sst_5 , also probably reflecting the stabilization of sst_2/sst_5
 37 heterodimers. Treatment with the sst_2/sst_5 -preferential compound BIM-23244 resulted in an
 38 even more pronounced effect compared to lanreotide (Fig. 4B). Compared with the
 39 constitutive levels, BIM-23244 did not affect the amount of sst_1/sst_2 heterodimers (Fig. 4A)
 40 and BIM-23704 did not affect that of sst_2/sst_5 heterodimers (Fig. 4B). Thus, the bi-specific
 41 sst_1/sst_2 and sst_2/sst_5 -preferential compounds enhanced the stabilization of the corresponding
 42 heterodimers.

43
 44 *Effect of SRIF analogs treatment on protein expression of p27^{Kip1}, p21 and cyclin E in LNCaP*
 45 *cells.* The effect of SRIF analogs on the expression of proteins involved in cell cycle arrest
 46 was then evaluated. Relative density data revealed that all SRIF analogs caused a significant
 47 induction in p27^{Kip1} and p21 and down-regulation of protein expression of cyclin E, compared
 48 to cells treated with media alone (fig. 5). In particular, BIM-23244 (sst_2/sst_5) caused 1.6- and
 49 2.4-fold increases of the protein expression of p27^{Kip1} and p21, respectively. as well as a
 50 significant decrement (-34%) in cyclin E level. BIM-23926 (sst_1) was able to increase p27^{Kip1}
 51 and p21 (1.74 and 2.63 fold. respectively) and reduce (-11%) cyclin E protein levels.

52
 53 *SRIF agonists down-regulate IGF-I and IGF-II secretion in LNCaP cells.* The IGF system is

1 known to contribute to PCa progression through systemic and paracrine/autocrine
2 mechanisms. Specifically, LNCaP cells were shown to secrete small amounts of IGFs. Thus,
3 we evaluated the ability of SRIF agonists to regulate IGF-I and -II secretion by these cells,
4 after a 48-h treatment. BIM-23244 ($ss_{2/5}$) and lanreotide significantly inhibited IGF-I (-
5 91% and -36%, respectively) and IGF-II (-59 and -68%, respectively) secretion. The
6 inhibitory effects of BIM-23244 on both IGFs and lanreotide on IGF-I were abolished by co-
7 treatment with the ss_{2} antagonist BIM-23627. Conversely, treatment with BIM-23704
8 ($ss_{1/2}$) produced a +33% increase of IGF-I and a -43% decrease of IGF-II secretion (Table
9 2), which were dramatically reduced in the presence of BIM-23627. Among the
10 monospecific ligands, BIM-23926 (ss_{1}) markedly reduced IGF-I (-85%) and IGF-II (-68%)
11 secretion, BIM-23120 (ss_{2}) significantly inhibited IGF-I (-79%) and slightly stimulated
12 (+19%) IGF-II secretion, BIM-23206 (ss_{5}) inhibited both IGF-I (-49%) and IGF-II (-86%)
13 secretion (Table 2). In conclusion, the secretion of IGFs by LNCaP cells appears to be
14 markedly affected by almost all SRIF analogs tested.

15
16 *Effect IGFs on SRIF agonists-induced inhibition of LNCaP cell proliferation.* In order to
17 study the role of IGFs on growth of PCa cells, we evaluated the effect of exogenous IGF-I
18 and IGF-II, tested alone or in combination with SRIF agonists, on LNCaP cell proliferation.
19 As shown in Table 3, a 48-h treatment with IGF-I alone significantly increased LNCaP cell
20 proliferation (+18%). Moreover, the inhibitory effects of lanreotide, BIM-23244, BIM-23120,
21 BIM-23206 and BIM-23926 was fully counteracted by the addition of IGF-I. On the contrary,
22 the administration of exogenous IGF-II did not change LNCaP cell proliferation, nor affected
23 the antiproliferative action of each SRIF agonist.

24
25 *SRIF agonists reduce the proteolytic activity of LNCaP conditioned media.* Another IGF
26 system component with relevance to the local control of PCa progression is IGFBP-3, which
27 binds IGF-I and also undergoes to degradation by proteolysis, resulting in lower IGF-I-
28 binding. Thus, we evaluated the ability of CM from LNCaP cells to cleave exogenous rh-
29 IGFBP-3 by WB. After a 5-h incubation with CM from untreated LNCaP cells, intact rh-
30 IGFBP-3 was dramatically reduced, compared to vehicle (PBS). Such extensive proteolytic
31 cleavage was partially reduced by treatment of LNCaP cells with SRIF analogs. Moreover,
32 combined treatment with the antagonist BIM-23627 and the mono-specific or bi-specific ss_{5}
33 analogs abolished the antiproteolytic effect induced by SRIF analogs (Fig. 4C). SRIF
34 analogs, therefore, seem able to interfere with the IGF system also by reducing the
35 proteolytic degradation of IGFBP-3.

36 37 **DISCUSSION**

38
39 The present study reports the substantial evidence for an important role of the SRIF system
40 in controlling PCa cell proliferation. Specifically, four ($ss_{1-2,3-5}$) out of five ss_{5} receptors
41 were found to be expressed in the LNCaP cell line, an *in vitro* model of human androgen-
42 dependent PCa. Their activation by selective SRIF agonists resulted in a significant anti-
43 proliferative effect with a peculiar pattern according to receptor subtype, ligand affinity and,
44 possibly, receptor dimerization. Moreover, such treatments were also able to modulate the
45 profile of the IGF system, known to be involved in PCa progression (Russell et al., 1998).
46 The ss_{5} expression profile observed in LNCaP cells partially differs from that reported in
47 other studies in the same cell line, which showed either the expression of all receptors (Liu et
48 al., 2008) or of some of them (Dizeyi et al., 2002; Taylor et al., 1994; Zapata et al., 2002).
49 Moreover, a great heterogeneity exists in the pattern of ss_{5} expression in other PCa cell lines
50 and normal prostate and PCa tissue (Halmos et al., 2000; Sinisi et al., 1997). Interestingly,
51 ss_{1} and ss_{3} were markedly up-regulated by different experimental conditions, an
52 observation in line with previous evidence of ss_{5} modulation by hormones. Indeed, it has
53 been recently shown that in PCa cells, treatment with 5-aza decitabine and trichostatin in

1 combination increased sst_5 mRNA content (Liu et al. 2008), similarly to the effects observed
2 in other cell systems after treatment with glucocorticoids or with estrogens (Djordjijevic et
3 al., 1998; Kimura et al., 2008; Viguerie et al., 1987; Visser-Wisselaar et al., 1996). It has
4 been also documented that hormone therapies may be responsible for a change in sst_s
5 expression in PCa (Reubi 2004). Thus, one may speculate that combined androgen blockade
6 therapy in PCa patients might possibly up-regulate the expression of some sst_s , thereby
7 improving some diagnostic applications (*i.e.*, *in vivo* scintigraphy by using radiolabelled-
8 SRIF analogs) (Khan et al., 2008), as well as the therapeutic efficacy of SRIF analogs.
9 Although few data exist about the selected antiproliferative effect of SRIF and related
10 agonists on PCa model systems (Bogden et al. 1990; Brevini et al., 1993), these studies did
11 not fully address the potential and selective role of the different sst_s in PCa progression.
12 Thus, we evaluated the effects of selective SRIF agonists on LNCaP cell growth. BIM-23244
13 (the sst_2/sst_5 preferential compound) and lanreotide (a sst_2 preferential compound with a
14 moderate affinity for sst_5) were significantly more potent in inhibiting LNCaP cell
15 proliferation than the mono-specific sst_2 - and sst_5 -preferring analogs (BIM-23120 and BIM-
16 23206, respectively), tested either alone or in combination. These differences between the
17 mono- and the bi-specific compounds might be due to the ability of BIM-23244 and, to a
18 lesser extent, of lanreotide to stabilize or enhance sst_2/sst_5 dimerization (corresponding to the
19 amount of sst_2 co-immunoprecipitated with sst_5), as suggested by the increased stabilization
20 (and/or number) of the sst_2/sst_5 dimers on the cell membrane after treatment with these
21 compounds (Fig. 4B). This finding is in agreement with the ability of sst_5 in regulating sst_2
22 trafficking (Sharif et al., 2007) and/or its retain within the membrane (Ben-Shlomo et al.,
23 2005), and might also explain the efficacy of BIM-23244 over a wider dose range compared
24 with lanreotide. Moreover, in contrast with the reversibility of the effect observed with
25 lanreotide, the increased activity of BIM-23244 was not counteracted by the sst_2 antagonist,
26 BIM-23267. Furthermore, in LNCaP cells, BIM-23244, in agreement with previous
27 observations in other cell systems, such as human pituitary adenoma and NSCLC Calu-6
28 cells (Ferone et al., 2005; Saveanu et al., 2001) closely mimicked the effects of native SRIF.
29 Differently from human medullary thyroid TT cells, where BIM-23206 completely
30 counteracts the suppressive activity of BIM-23120 (Zatelli et al., 2001), in LNCaP cells the
31 effects of these agonists were both directed toward a significant antiproliferative effect,
32 suggesting not only tissue-specific activity, but also a tissue-specific interactions between
33 these two sst_s . The different cross-talk between sst_s in different districts may partially explain
34 the functional tissue-specificity of an ubiquitous hormone such as SRIF. Dose-response
35 experiments showed that the sst_1 -preferential compound BIM-23296 negatively affected cell
36 proliferation, with a greater potency than sst_2 and sst_5 agonist, possibly due to sst_1 up-
37 regulation and/or to a low agonist-induced internalization of sst_1 (Hofland et al., 2003; Nouel
38 et al., 1997; Sarret et al., 1999). Notably, sst_1 was found to be the most abundantly expressed
39 SRIF receptor in PCa tissue (Halmos et al., 2000; Reubi et al., 1995). The results obtained
40 with the sst_1/sst_2 -preferential agonist BIM-23704 suggest that the simultaneous recruitment
41 of these two receptors, although able to stabilize sst_1/sst_2 dimerization, is less effective in
42 terms of antiproliferation and fully sensitive to specific sst_2 antagonism, than sst_2/sst_5
43 activation.
44 Moreover, our data suggest that the antiproliferative effects of these SRIF agonists on
45 LNCaP cells, may, at least in part, result from cell cycle arrest in the G_0/G_1 phase, as
46 suggested by the observed up-regulation of the cyclin-dependent kinase inhibitors p27 and
47 p21 and the inhibition of cyclin E. Along with these direct actions on cell cycle, SRIF may
48 also regulate tumor growth by indirect action suppressing the secretion of growth-promoting
49 hormones and growth factors (Lamberts et al., 2002). In addition to systemic suppression of
50 the GH-IGF-I axis, SRIF analogs may also affect paracrine and autocrine IGF-I loops within
51 the tumoral prostatic tissue (Pietrzkowski et al., 1993). LNCaP cells have been shown to
52 secrete low amounts of IGF-I and IGF-II and to express some IGF-binding proteins
53 (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 ($ss_1/2$ 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 $ss_2/5$ -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/antisecretive actions represent a set of complementary favorable events in
51 terms of antiproliferative activity. Moreover, the bi-specific $ss_2/5$ -preferential compound
52 BIM-23244-promoted enhancing of $ss_2/5$ 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 sst_1/sst_2 appears however less effective than
2 that of either sst_1 alone or sst_2/sst_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.

5

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7

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13

14

15 **FIGURE LEGENDS**

16

17 **Fig. 1.** *RT-PCR and Western blot analysis of sst₁₋₅ expression in LNCaP cells.* A) Total RNA
 18 extracts from LNCaP cells, non-functional pituitary adenoma (NFPA - sst_{1-2A-3-5} positive
 19 control) and benign prostatic hyperplasia (BPH - sst₄ positive control) samples were subjected
 20 to RT-PCR analysis using oligoprimers specific for sst₁₋₅ and 18 s ribosomal protein (internal
 21 control); B) Cell extracts were subjected to Western blot analysis using antisera against sst₁,
 22 sst_{2A}, sst₃, sst₄ and sst₅. The human Calu-6 NSCLC cell line and a sample of benign prostatic
 23 hyperplasia (BPH) were included as positive controls.

24

25 **Fig. 2.** *Influence of different culture conditions on sst₁ and sst₃ gene and protein levels of*
 26 *expression in LNCaP cells.* LNCaP cells were cultured either with 10% FBS (standard
 27 culture conditions) or with 2% FBS (experimental culture conditions) for 48 h. A)
 28 Semiquantitative RT-PCR analysis of sst₁ and sst₃ gene expression. Parallel amplification of
 29 18 s ribosomal protein mRNA served as internal control. B) Real-time RT-PCR analysis of
 30 sst₁ and sst₃ gene expression. Data are presented as mean fold increment \pm SEM, n = 3, **P
 31 < 0.01 vs. 10% FBS. C) Western blot analysis of sst₁ and sst₃ in protein extracts from LNCaP
 32 cells.

33

34 **Fig. 3.** *Effects of SRIF analogs and sst₂-selective antagonist BIM-23627 on BrdU-*
 35 *incorporation on LNCaP cells.* Mean dose-response growth-inhibition curves obtained with
 36 LNCaP cells cultured in 96-well plates for 48 h with: A) lanreotide (Lan), bi-specific
 37 sst₂/sst₅-preferential compound BIM-23244 and SRIF; B) sst₂-preferential compound BIM-
 38 23120, sst₅-preferential compound BIM-23206 and the combination of both; C) sst₁-
 39 preferential compound BIM-23296 and bi-specific sst₁/sst₂-preferential compound BIM-
 40 23704; D) Lan (10⁻⁷ - 10⁻¹¹ M) with or without BIM-23627 (Ant; 10⁻⁷ M) and E) BIM-23704
 41 with or without Ant (10⁻⁷ M). Control wells were treated with vehicle solution. Results are
 42 expressed as mean (\pm SE) % growth inhibition vs. control (-), n = 8.

43

1 **Fig. 4.** *Sst*₁/*sst*₂, *sst*₂/*sst*₅ heterodimerization and effect of SRIF analogs on rh-IGFBP-3
2 proteolytic activity of LNCaP cell conditioned medium. To assess the stabilization of *sst*₁/*sst*₂-
3 and *sst*₂/*sst*₅-driven heterodimerization, membrane preparations from LNCaP cells were,
4 respectively: A) immunoprecipitated with anti-*sst*₂ antibody and immunoblotted with anti-*sst*₁
5 antibody. The band of 60 kDa corresponds to the *sst*₁ co-immunoprecipitated with *sst*₂; B)
6 immunoprecipitated with anti-*sst*₅ antibody and immunoblotted with anti-*sst*₂ antibody. The
7 band of 45 kDa corresponds to the *sst*₂ co-immunoprecipitated with *sst*₅; C) Exogenous rh-
8 IGFBP-3 was incubated for 5 h at 37°C with conditioned media from LNCaP cultured in the
9 presence or absence of different SRIF analogs (10^{-9} M) and *sst*₂-selective antagonist BIM-
10 23627 (10^{-7} M). As internal control exogenous rh-IGFBP-3 was incubated only with vehicle
11 (PBS). The amount of residual intact rh-IGFBP-3 was visualized by immunoblotting.
12 Lanreotide and BIM-23627 are indicated as Lan and Ant, respectively.
13 IP: immunoprecipitation; WB: Western blot
14

15 **Fig. 5.** Effect of SRIF analogs treatment on protein expression of *p27^{Kip1}*, *p21* and *cyclin E* in
16 LNCaP cells. As detailed in the “Materials and Methods” section, the cells were treated for
17 48 h with SRIF analogs at the concentration of 10^{-9} M and vehicle alone (control). Total cell
18 lysates were prepared for immunoblot analysis. The values above the figures represent the
19 relative density of each band normalized to tubulin. The data shown here are from a
20 representative experiment repeated three times with similar results.
21

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

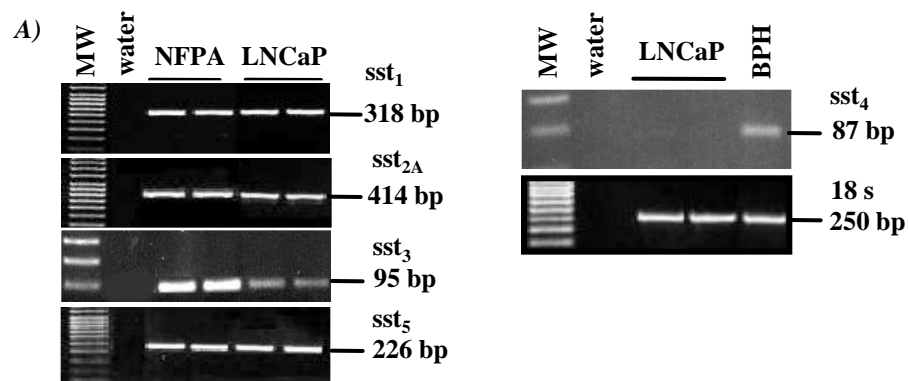
C	IGF-II	Lan	Lan + IGF-II	BIM-23244	BIM-23244 + IGF-II	BIM-23704	BIM-23704 + IGF-II	BIM-23206	BIM-23206 + IGF-II	BIM23926	BIM23926 + IGF-II
	2.4 ± 7.55	-23.1 ± 2.9	-16.6 ± 3.4	-20.8 ± 1.8	-17.5 ± 3.5	-13.1 ± 0.9	-9.5 ± 0.5	-12 ± 2.0	-8.8 ± 1.1	-13.3 ± 1.9	-9.1 ± 2.9

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)

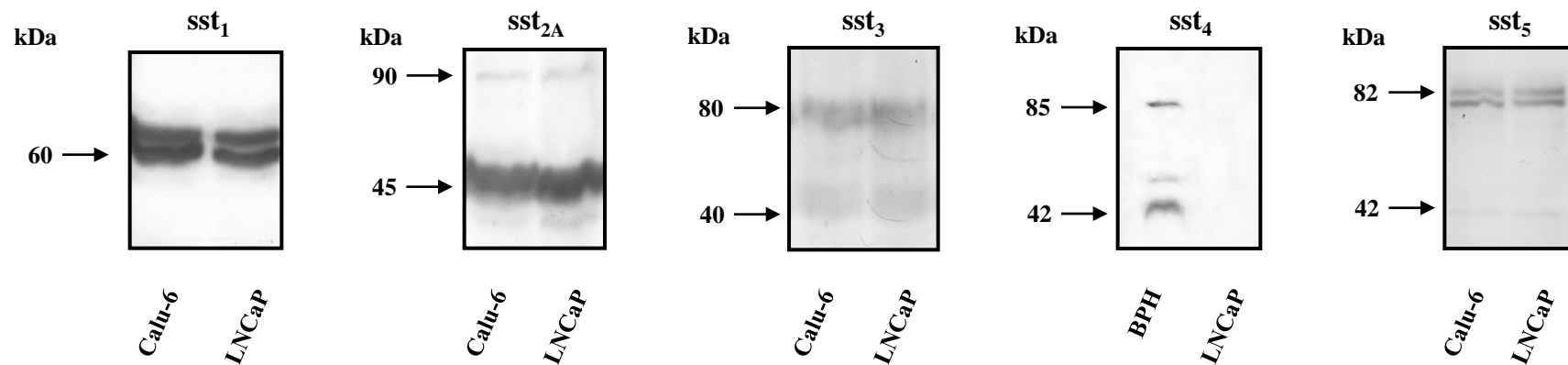


Fig. 2

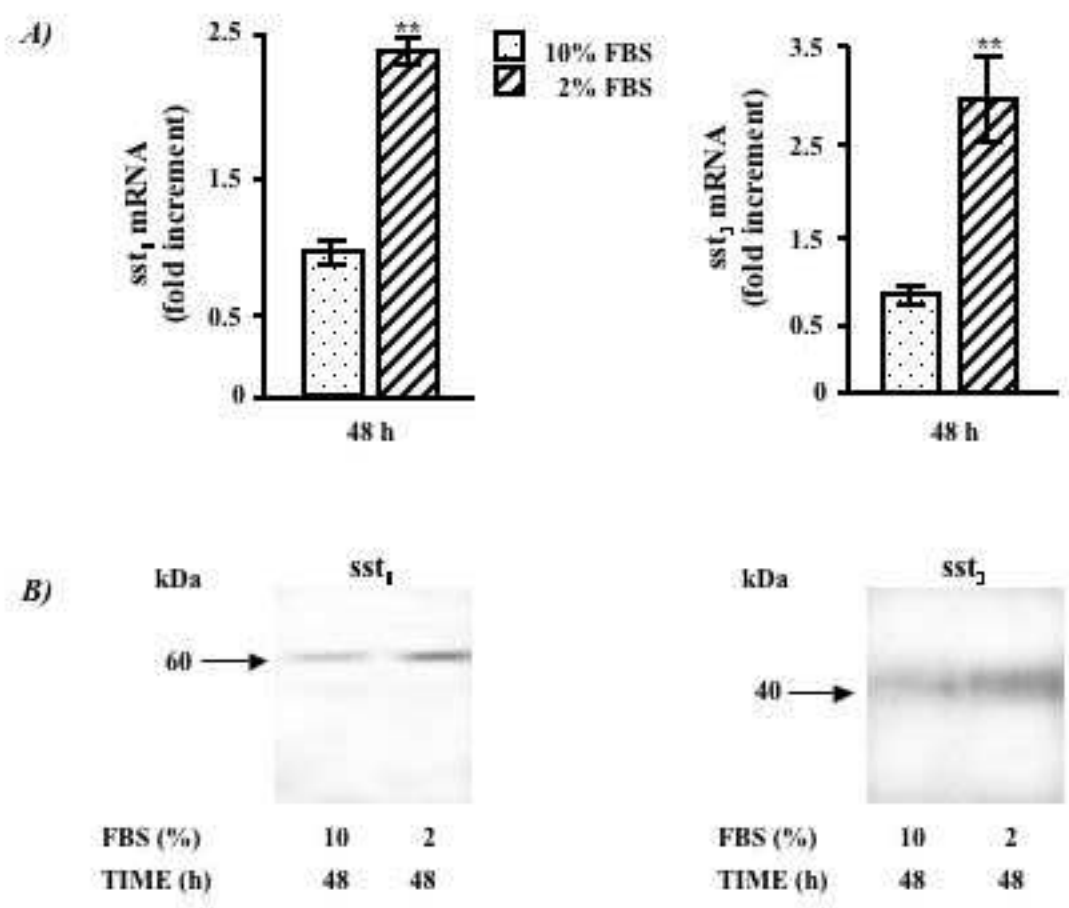


Fig. 3

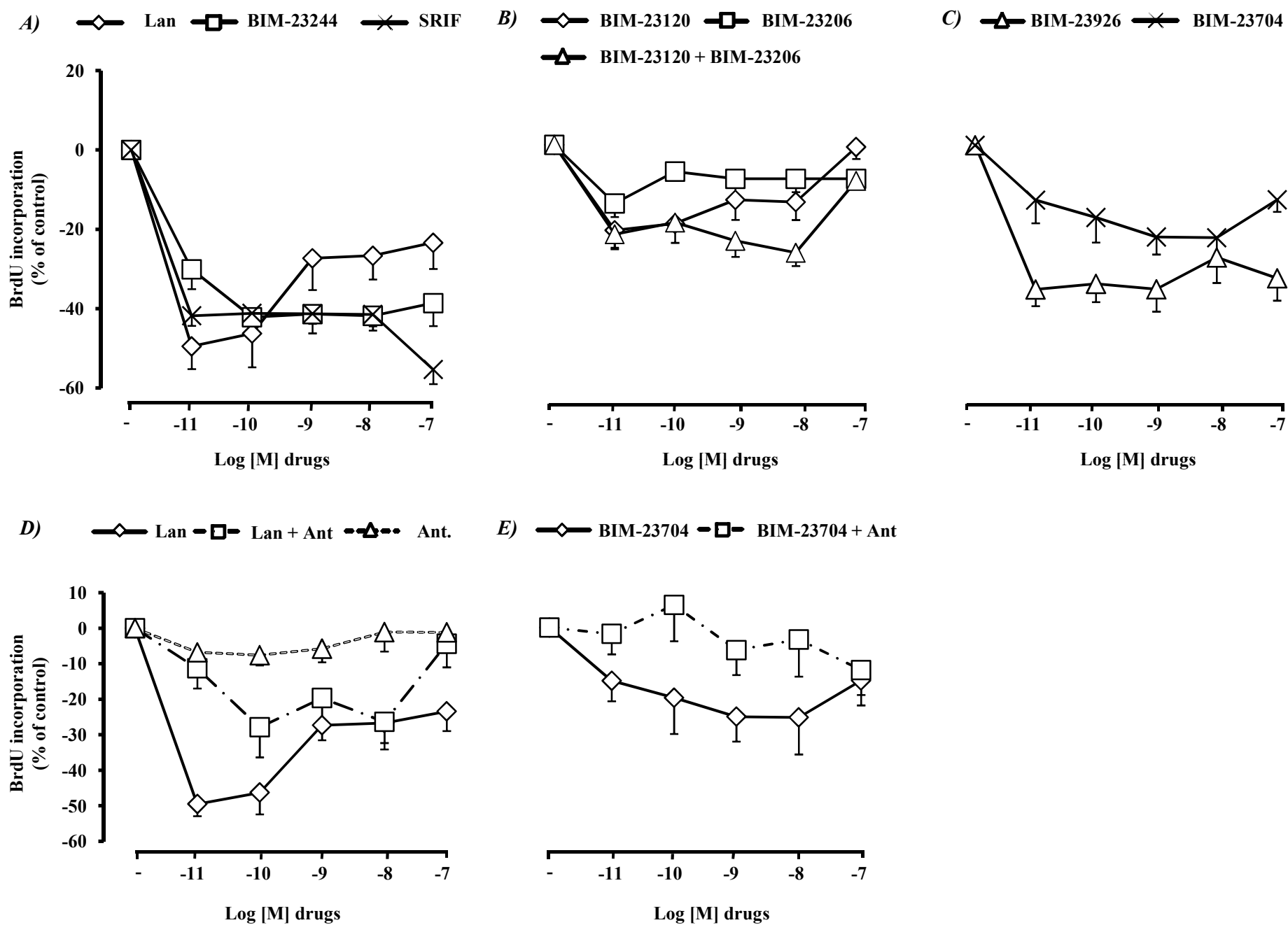


Fig 4

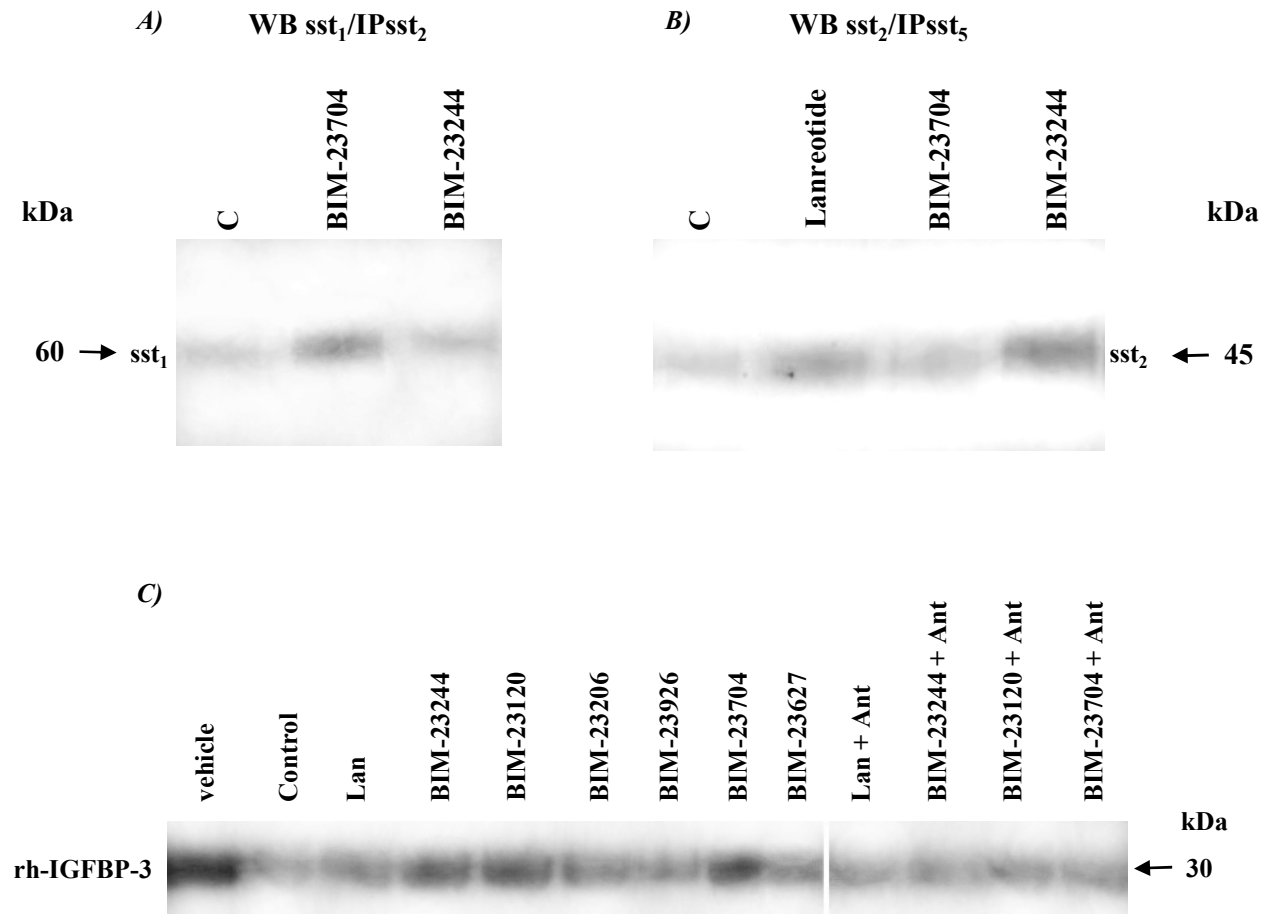


Figure 5

Fig. 5

