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Dexamethasone plus Octreotide Regimen Increases Anticancer Effects of Docetaxel on TRAMP-C1 Prostate Cancer Model

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Abstract. *Aim: The aim of this study was to evaluate whether the neoadjuvant use of the dexamethasone (DEX) plus octreotide (OCT) regimen can improve the direct anticancer effects of docetaxel (DOC) in the TRAMP-C1 prostate cancer model. Materials and Methods: TRAMP-C1 cells were first characterized for the expression of SSTR1-5 and then were inoculated onto the femur of C57Bl mice. Investigation protocols employed TRAMP-C1 cell proliferation and invasion assays, analysis of radiographic images of the bone lesions and overall survival of the diseased animals. Results: The triple combination treatment scheme showed significant anticancer effects, in both proliferation and invasion assays, compared to any single agent treatment scheme. DOC treatment following the neoadjuvant administration of DEX plus OCT regimen improved significantly the anticancer effects both on the grading of the bone lesions and on the overall survival of the diseased animals. Conclusion: Our data suggest that the neoadjuvant administration of DEX plus OCT regimen can improve the anticancer effects of DOC on the TRAMP-C1 model.*

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The presence and more importantly the increased number of bony lesions defines poor clinical response to androgen ablation therapies, short progression-free survival to medical or surgical castration, limited response to chemotherapy and poor survival in advanced prostate cancer patients (1-9). The vast majority of bony lesions in prostate cancer are mainly of blastic nature, thus implicating the production of prostate cancer cell-derived mitogen(s) capable of stimulating woven bone production locally (9-12). An extensive line of research has documented that bones constitute a favorable microenvironment for homing prostate cancer cells (13, 14). Indeed, metastatic prostate cancer cells are capable of expressing high levels of urokinase-type plasminogen activator (uPA), which can orchestrate a sustained hydrolysis of several extracellular growth factor-binding proteins, such as the insulin-like growth factor-binding proteins (IGFBPs), thereby increasing the local bioavailability of IGFs, interleukin 6 (IL-6) and transforming growth factor beta 1 (TGFβ1) (14-16).

Moreover, bone microenvironment-related growth substances can stimulate the expression and secretion of osteoprotegerin (OPG) by the prostate cancer cells, which in turn inhibits osteoclastogenesis, thus favouring the blastic reaction at the sites of bone metastasis (17, 18). These cell-cell interactions occurring within the bone metastasis microenvironment can also confer antiapoptotic/survival effects on prostate cancer cells, thus conferring bone microenvironment-mediated resistance to androgen ablation-induced and chemotherapy-induced apoptosis (13, 17, 19-21).

Interestingly, the activation of glucocorticoid receptor (GR) suppresses the expression of uPA, IGF-1, TGFβ1 and IL-6 of prostate cancer cells and of osteoblasts, while it

exerts a direct inhibition of the growth of prostate cancer cells and osteoblasts *in vitro* (22-26). In addition, somatostatin (SM) and its receptors (SSTR1 to -5), although they have a wide distribution among several tissues, predominantly inhibit the secretion of growth hormone (GH) at the pituitary level (27). However, SM and SSTRs appear to have an important role in cancer biology, including prostate cancer (28, 29). Therefore, the synthesis of long-acting SM analogs, such as octreotide (OCT) and lanreotide (LAR), both acting *via* SSTR2 (mainly) and SSTR5, engaged them in the treatment of GH-secreting pituitary adenomas and neuroendocrine gastrointestinal tumours (29, 30-31). Interestingly, a part of the direct antitumor effect of these SM-analogs *via* SSTR2 and SSTR5 can also influence tumour growth *via* the systemic and/or local reduction of the GH-dependent IGF production (32).

These data enabled us to design a novel combination therapy for advanced prostate cancer, using the combination treatment of dexamethasone (DEX) plus SM analog, targeting the host tissue (bone) microenvironment-related survival factors, such as IGF-1, IL-6, TGFβ1 and uPA, in the clinical setting of castration-resistant prostate cancer (33-35). Indeed, such combination treatment produced significant clinical responses in castration resistant prostate cancer bearing diffused bone lesions (36-40).

This therapeutic approach has never been tested in combination with chemotherapy, particularly with docetaxel (DOC), which is considered the standard therapy for castration resistant prostate cancer (41-43). Therefore, we designed the present study, starting by the characterization of SSTR expression in transgenic adenocarcinoma mouse prostate cancer cells (TRAMP-C1) and by the development of TRAMP-C1 cell-induced bone lesions onto the femur of C57Bl mice. Then we proceeded in the evaluation of the anticancer effects of DEX, OCT and DOC, using single treatment schemes and double or triple combination treatment schemes both *in vitro* and *in vivo*. The aim of this study was to evaluate whether the neoadjuvant use of DEX plus OCT regimen can improve the direct anticancer effects of DOC in the TRAMP-C1 model.

Materials and Methods

Cell cultures. TRAMP-C1 cells were propagated from the transgenic mouse prostate (TRAMP), which was developed as a model for studying tumorigenesis, androgen-independent prostate cancer growth and metastatic processes of prostate cancer (44-47). The TRAMP-C1 cell line was obtained from the American Type Culture Collection (ATCC catalogue no.CRL-2730, Manassas, VA, USA) and TRAMP-C1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen/Gibco, Grand Island, NY, USA), containing 5% fetal bovine serum (FBS, Invitrogen/Gibco, Grand Island, NY, USA), 5% fetal calf NuSerum (Becton Dickinson Biosciences, Bedford, MA, USA), 5 μg/ml insulin (Biochemika, Sigma–Aldrich, Steinheim, Switzerland), 10 nM dehydrolsoandrosterone (Sigma Chemical Co, St. Louis, MO, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen/ Gibco, Grand Island, NY, USA). TRAMP-C1 cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Proliferation and cytotoxic assays. The cytostatic and cytotoxic effects of the compounds were assessed on TRAMP-C1 cells, *in vitro*. The TRAMP-C1 cells were cultured at a density of 1x104 cells/ml in culture medium, and the cultures were maintained for 72 h in an incubator at 37˚C with 5% CO_2. Twenty four hours later, the cells were treated with the following concentration ranges: DEX (0.1-160), OCT (2.5-250) and DOC (0.0001-1.5) (μg/ml final concentration) for 48 h. The viability of cultured cells was estimated by the sulforhodamine B colorimetric assay (SRB), as previously described (48, 49). In brief, TRAMP-C1 cells were split into 96-well plates. After incubation, anchorage-dependent cells were directly fixed by the slow addition of 50 μl of 50% trichloroacetic acid solution/well and fixation proceeded for 30 min at 4˚C. After fixation, plates were washed five times with tap water, and were air dried. One hundred microliters of SRB solution (0.4% in 1% acetic acid) were added to each well of the 96-well microplates and staining was carried out at room temperature for 30 min. Residual dye was washed out with 1% acetic acid and plates were air dried. To each well, 100 μl of Tris base solution (10 mM, pH 10.5) was added. The optical density (O.D.) was measured in a microtiter plate reader (Anthos Labtec HT2, version 1.06, Salzburg, Austria) at 540 nm. Each drug concentration was tested in sextuplicate experiments. The coefficient of variation (CV) for all experimental tests was found to be <10%.

For each agent under investigation, a dose-dependent effect curve was prepared. The inhibition of cell growth was expressed as the fraction (Fu) of TRAMP-C1 cells which remained unaffected (survival fraction: SF) and was estimated relative to the percentage of control cells, derived from the following equation: Fu=VCx/VCc, where VCx and VCc represent the experimental and the control viable cell numbers, respectively. Drug potency was expressed as IC_{50} values (concentration of each drug that produces 50% inhibition on cell growth) and was calculated from the plotted dose-effect curves by least-square regression analysis.

TRAMP-C1 cells were also treated with the following drug concentration treatment schemes, using ratio of DEX:OCT (2:1 and 1:1) and DEX:OCT:DOC (7000:13330:1; 5000:10000:1; 2500:5000:1; 1000: 2500:1) (μg/ml). The combined drug interaction was assessed by median effect analysis as follows, using the Chou and Talalay's median effect analysis and combination index (CI) method (50). Accordingly, when the CI<1, CI=1 and CI>1, the result was considered to be a synergistic effect, an additive effect, or an antagonistic effect, respectively. The interaction of the agent was quantitated by the CI method across the entire range of the dosing effects. CI values were calculated with CalcuSyn Software (Biosoft, Cambridge, UK) as a function of the level of antiproliferative activity (51, 52). Data generated from the CI method were used to quantify the dose-reduction index (DRI) for the double or triple combination treatments schemes (two or three drugs). The DRI represents a measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the dose of each drug alone. Each CI or DRI ratio was represented by its mean value derived from at least five independent experiments. The DRI was measured by comparing the doses required to reach a given degree of inhibition (50% growth inhibition) when using the drug as a single agent and in combination treatment schemes.

Cell invasion assay. Cell invasion analyses were performed as described previously (53). Transwell basement membrane-coated inserts (Cell Biolabs cytoselect™ , invasion assay Cat. No CBA-100-C, San Diego, CA, USA) were used for invasion assay. As chemoattractant FBS was added to the lower chamber. TRAMP-C1 cells (1×10^6 cells/ml) were suspended in DMEM (serum-free) containing the compounds under investigation, alone and in combination, as follows: DEX (110 μg/ml; final concentration), OCT (200 μg/ml; final concentration), DEX plus OCT (29.3:26.3; μg/ml; final concentration), DOC (0.015 μg/ml; final concentration), and DEX plus OCT plus DOC (15.4:38.9:0.015; μg/ml; final concentration) to the upper chamber and incubated for 24 h. After the incubation period, cells that had passed through the membrane were stained with Cell Stain Solution (0.1% crystal violet) and the results were assessed using a 96-well format plate reader, measuring the absorbance at a wavelength of 595 nm.

TRAMP-C1 Cell-induced bone lesions, in vivo. C57Bl male mice were employed for the development of TRAMP-C1 cell-induced bone lesions and subsequently they were used for the evaluation of the antitumor activity of the compounds under investigation *in vivo*. These mice were obtained from the experimental section of the Research Center at Theagenion Cancer Hospital, and were kept under controlled temperature and humidity conditions, in sterile cages, with access to food and water *ad libitum* and on a 12- light/dark cycle. The experimental animal production laboratory follows the institutional and national guidelines for the care and use of laboratory animals.

TRAMP-C1 cells were cultured in monolayer cultures and after reaching confluence of 80%, the cells were trypsinized, harvested, centrifuged, washed with serum-free medium and suspended in the same medium. At day 0 of the experimental protocol, 1×10^5 TRAMP-C1 cells in suspension were injected/inoculated directly onto the periosteum of the femur (IF), using total inoculation volume of 0.2 ml, of 8 to10-week-old male C57Bl mice. The TRAMP-C1 cells were then left to grow at the inoculation site and the bone lesions were weekly monitored by radiography. A total number of 32 mice were inoculated with TRAMP-C1 cells. Approximately 42 days after the initial inoculation, when the average size of the tumor at the inoculation sites on the femur was approximately 1 cm^3, the mice were separated into six experimental groups with 5 mice/group for each treatment scheme and 7 mice for the diseased control group/placebo group. The animals were randomly assigned to treatment protocols with: DEX (G.A. Pharmaceuticals S.A. Athens, Greece) administered by subcutaneous injections (*s.c.*) at a dose of 0.1 mg/day from day 42 up to day 49; OCT (Sandostatin-LAR 30 mg; Novartis Pharma AG, Basle, Switzerland) was administered by intramuscular (i.m) injections at a dose of 20 mg/kg on day 42 of the protocol and DOC (Sanofi-Aventis Pharma, Essex, UK) was administered by subcutaneous (*s.c.*) injections at a dose of 10 mg/kg on day 49 of the protocol. We administered single (DEX or OCT or DOC), double (DEX plus OCT) and triple (DEX plus OCT plus DOC) combination treatment schemes using an identical administration treatment protocol with regards to time schedule and drug dosages.

Evaluation of the in vivo anticancer effects. The antitumor activity was assessed using the median survival time (MST; days) of diseased animals treated with each treatment scheme (T) relative to the one of diseased animals receiving normal saline injections (C) as % MST and reported as T/C (%). According to the NCI (USA) criteria, a significant anticancer effect must result in a T/C higher than 125% (54). In addition, in order to evaluate the effects of treatment schemes on bone lesions, we analyzed the images of the bone lesions obtained at the time of death. Images of the inoculation sites of the experimental mice were recorded by a digital mammographic system (General Electric Medical System/GE, Carquefou-Nantes, France, and Senographe DMR+, Buc Cedex, France) by applying 38 kV and 14 mAs and creating images on digital films (Regius Model 190/Drypro Model 793, Konica Minolta, Shinjuku-ku, Tokyo, Japan). Such images were further analyzed as previously described (55). The images were evaluated by three different individuals, including one radiologist, none of whom had previous knowledge of the experimental procedure/protocol. The semiquantitative analysis of the images resulted in a grading system modificated by our group from a previous study, (55) as follows: grade A=development of soft tissue tumor with metastasis to distant organs, without affecting the femur (inoculation site); the respective femoral image was normal when compared to the one of the contralateral femur; grade B=development of soft tissue tumor with metastasis to distant organs and radiographic images of asymmetric bony lesions limited to the inoculation site; grade C=development of soft tissue tumor with metastasis to distant organs and presence of significant bony lesions with major peripheral margin breaks and bone surface disruption.

Measurements of serum levels of IGF-1, receptor activator of NFκβ (RANK) ligand (RANKL), OPG and IL-6. Blood samples were collected from the diseased animal group receiving no treatment (placebo group) at day 30 after the development of tumors at the inoculation site and from 7 healthy animals of the same age (healthy controls). Blood was centrifuged at 2,000 ×*g* for 10 min and serum was stored at –80˚C until measurement. The concentration of soluble markers under investigation was determined using Mouse/Rat IGF-1 Quantikine ELISA kit, TRANCE/RANKL/TNFSF11 Quantikine ELISA kit, mouse OPG/TNFRSF11B, and mouse IL-6 Quantikine ELISA kit, all from R&D System (Minneapolis, MN, USA). The absorbance of the samples was measured using an automated microplate reader (Anthos Labtec HT2, version 1.06) and the serum levels were calculated according to the standard curves developed following the manufacter's instructions. Limits of assay detection for IGF-I: 0-2000 pg/ml (sensitivity- mean minimum detectable dose (MDD): 3.5 pg/ml), for RANKL: 0-2000 pg/ml (mean MDD: less than 5 pg/ml, for OPG: 0-2000 pg/ml (mean MDD: 4.5 pg/ml) and for IL-6: 0-50.0 pg/ml (mean MDD: 1.6 pg/ml). All samples were run in triplicate, the measurements and the results were expressed as mean values +/– standard deviation (SD).

RNA extraction, isolation and reverse transcription polymerase chain reaction (RT-PCR) analysis. Total RNA from TRAMP-C1 cells was extracted using TRI-Reagent (# RT-118; MRC, Cincinnati, OH, USA). First-strand cDNA was synthesized from 2 μg of RNA (TRAMPC1 cells) mixed with oligo dT18 (# SO132; Fermentas INC, Glen Burnie, Maryland, USA) and filled up to 12 μl with DEPC-treated ddH_2O. The reactions were then heated to 70˚C for 5 min and quick-chilled on iced water. Pre-Mixed dNTPs (10 mM each) (# SB-25U; HT BIOTECHNOLOGY Ltd., Cambridge, UK) and the reverse transcriptase (RT) buffer containing 200 U/μl of Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase, RNase H^- (# F-572L; FINZZYMES, Vantaa, Finland) RT were then added and the reactants were incubated at 37˚C for 60 min. The

Table I. *Primers used for the detection of somatostatin receptor mRNA (mSSTRs).*

Forward primer	Reverse primer
mSSTR1 5'-GCG CTG GTT GGT GGG CTT CGT-3'	5'-TTC AGG GCA GTG GCA TAG TAG TCG-3'
mSSTR2 5'-CTT GGC CAT GCA GGT GGC GCT AGT-3'	5'-ATG GGG TTG GCG CAG CTG TTG-3'
mSSTR3 5'-GCC CAT CAG TGA CCA GTG TCT ATA-3'	5'-GAA TGC GAC GTG ATG GTC TTA GCA-3'
mSSTR4 5'-AGA CAT GAA CGC GCC AGC AAC T-3'	5'-AGC TGG CCT GGT GTC AGC GAA G-3'
mSSTR5 5'-TAG TGC CTG TGC TCT ACT TGT TGG T-3'	5'-CAA AGC CTG CTG GTC TGC ATG AGC-3'

M-MuLV was inactivated at 70˚C for 5 min. The cDNA was amplified by polymerase chain reaction (PCR) with 200 nM of specific primers describe previously (56). The PCR reactions were performed using Taq PCR MasterMIX kit (#201445; QIAGEN, Crawley, West Sussex, UK). Each reaction comprised a 40 cycle program (94˚C for 30 min, 58˚C for 15sec and 72˚C for 3min). After amplification, PCR samples were run on 2% agarose gels (Bio-Rad, Hercules, CA, USA) and the products were visualized by ethidium bromide staining.

Western blotting. The TRAMP-C1 cells were washed with cold Phosphate Buffered Saline (PBS) and solubilized in lysis buffer and then cell lysates were boiled in sodium dodecyl sulfate (SDS) sample buffer, containing 0.5 M β-mercaptoethanol. Samples were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes for 2 h at 120 Volts. The membranes were blocked with 5% milk in Tris Buffered Saline with 0.1 % Tween 20 (TBS/Tween) (20mM Tris-(hydroxymethyl)-aminomethane buffer, 130 mM NaCl, pH: 7.6), and immunoblotted with primary antibodies for about 13 h at 4˚C using the primary antibody at 1:1000 titer in 1% milk (SSTR2 and SSTR5 antibodies, Cat Nub AB5575/AB 5681; Chemicon International, Temecula, CA,USA). Then membranes were washed 3 times for 5 min in TBS-T buffer and were incubated for 1 h at room temperature with secondary antibodies (1:2000 in 1% milk (anti-rabbit) Cat Nub AP132P; Chemicon). The proteins were visualized with horseradish peroxidate (HRP).

Statistical analysis. Student's *t*-test was used for comparison of the level of significance between the experimental groups. Differences with $p<0.05$ were considered significant. Microsoft Excel was used to compile data and to create corresponding graphs and tables.

Results

Characterization of the SSTRs in TRAMP-C1 cells. Since our experimental design included the use of OCT in a TRAMP-C1 model, we characterized TRAMP-C1 cells for the expression of the SSTRs. Using specific primers for the SSTRs (Table I), we detected the expression of the *SSTR1, SSTR2, SSTR3* and *SSTR5* mRNA in TRAMP-C1 cells (Figure 1A). SSTR4 was not detected in TRAMP-C1 cells, under our experimental conditions. Knowing that OCT acts mainly on SSTR2 and SSTR5, we proceeded to the verification of the expression of SSTR2 and SSTR5 at protein levels using western analysis (Figure 1B). Indeed, we documented the expression of SSTR2 and SSTR5, suggesting that OCT can affect the growth of the TRAMP-C1 model, directly.

Growth and toxicity assays. Our initial experiments sought to determine the responsiveness of TRAMP-C1 cells to each of the individual agents under investigation (DEX, OCT and DOC). Composite dose response curves were fit to the data using Excel software and drug concentrations that were able to produce 50% inhibition of the growth of TRAMP-C1 cells were determined. The IC_{50} values for DEX and OCT were 120g/ml and 150 μg/ml, respectively. The IC_{50} of DOC was 0.007 μg/ml ($p<0.001$) (Figure 2).

Analysis of the possible synergistic/additive anticancer effects of DEX, OCT and DOC on TRAMP-C1 cells were sought by using the combination treatment schemes. Our data enabled the calculation of the composite dose effect curves, median effects and CI plots (Figure 3). These results revealed that the combination of DEX plus OCT was moderately synergistic over a wide range of doses having a CI=0.677-0.882 and 0.590-0.820 at 2:1 and 1:1 concentration ratios, respectively. The treatment scheme of DEX plus OCT plus DOC (7000:13330:1; 5000:10000:1; 2500:5000:1; 1000:2500:1) produced an additive-synergistic effect (CI=0.552-0.988) at most ratios tested, and resulted in a strong inhibition of the TRAMP-C1 cell growth (Figure 4). Interestingly, the DRI analysis documented a considerable reduction by 5.071-fold of the DOC dose necessary to produce maximum inhibition of TRAMP-C1 cells *in vitro*.

Analysis of TRAMP-C1 cell invasion capability. To investigate the effects of DEX, OCT and DOC and their respective combination treatment schemes on metastatic capabilities of TRAMP-C1 cells, we used cell invasion assay. The analysis revealed that all the compounds and their combinations were able to reduce the invasion capability of the TRAMP-C1 prostate cancer cells (Figure 5). Single treatment schemes revealed that the invasion capacity of TRAMP-C1 cells was inhibited by 7.8% using DEX, by 25.5% using OCT, and by 13.2% using DOC single- agent

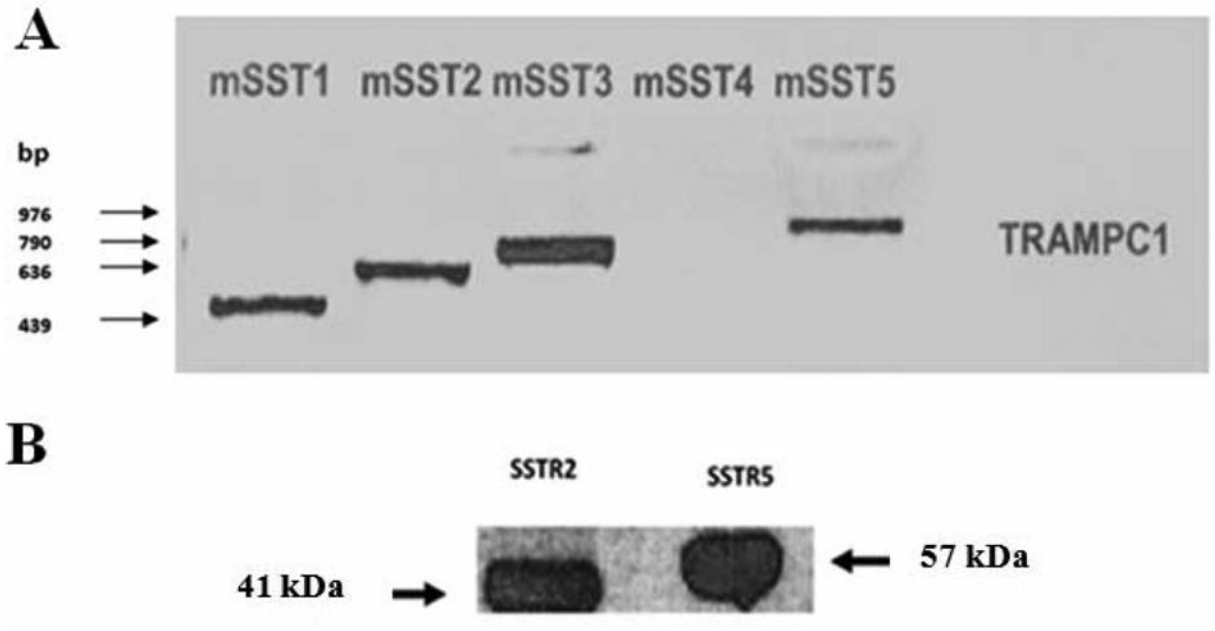

Figure 1. *The expression of somatostatin receptor (SSTRs) in TRAMP-C1 cells. A: Detection of the expression of SSTR sybtype mRNAs using reverse transcriptase-PCR. Note that we were unable to detect the expression of the SSTR4 in TRAMP-C1 cells, under our experimental conditions. B: Western analysis confirmed the expression of SSTR2 and SSTR5 at protein levels in TRAMP-C1 cells, suggesting that the administration of octreotide can act directly on TRAMP-C1 cells.*

treatment schemes. However, the triple combination treatment scheme resulted in inhibition by 37.3% of the TRAMP-C1 cell invasion capability (Figure 5).

Analysis of in vivo anticancer effects. The median survival time of the placebo group was 100 days. Based on the NCI criteria, the minimum increase of T/C (%), defining a significant anticancer activity is anything >125%. Our data showed that the single-agent treatment schemes and the combination treatment schemes, using DEX and OCT, did not meet the NCI criteria (>125%) for there being a significant effect on the overall survival in the TRAMP-C1 cell-induced model (Table II). Notably, the combination treatment scheme of DEX plus OCT achieved marginal NCI significance for anticancer effect, having a T/C value of 122%, as compared to the placebo group. With regards to the single DOC treatment scheme, our data revealed a T/C value of 133% ($p<0.01$) with respect to overall survival (Table II). Interestingly, the neoadjuvant use of DEX plus OCT regimen prior to DOC treatment produced further improvement of the lifespan of diseased animal with a T/C of 150% ($p<0.01$) as compared to DOC-treated animals and to the placebo group (Table II).

In addition, the radiographic analysis of the bony lesions revealed that such lesions produced evidence of blastic nature (Figure 6A). Furthermore, the analysis of the bone lesions using our grading system revealed that all the treatment schemes reduced the severity of bone lesions, as compared to the placebo group (Figure 6B). In the placebo group, imaging revealed bone lesions of grade C (100%) of animals. The single DOC treatment scheme reduced the bone

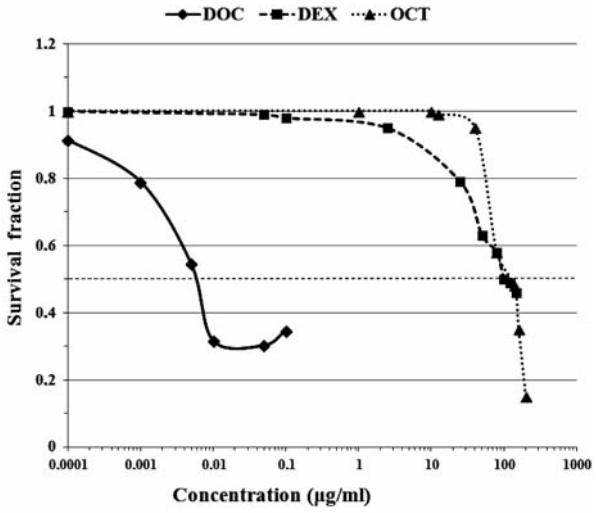

Figure 2. *The anticancer effects of dexamethasone (DEX), octreotide (OCT) and docetaxel (DOC) in TRAMP-C1 cells in vitro. The viability of cultured cells was estimated by the sulforodamine B colorimetric assay, as previously described (48-49). The IC50 values for DEX, OCT and DOC were calculated as being 120 µg/ml, 150 µg/ml, and 0.007 µg/ml ($p<0.001$), respectively.*

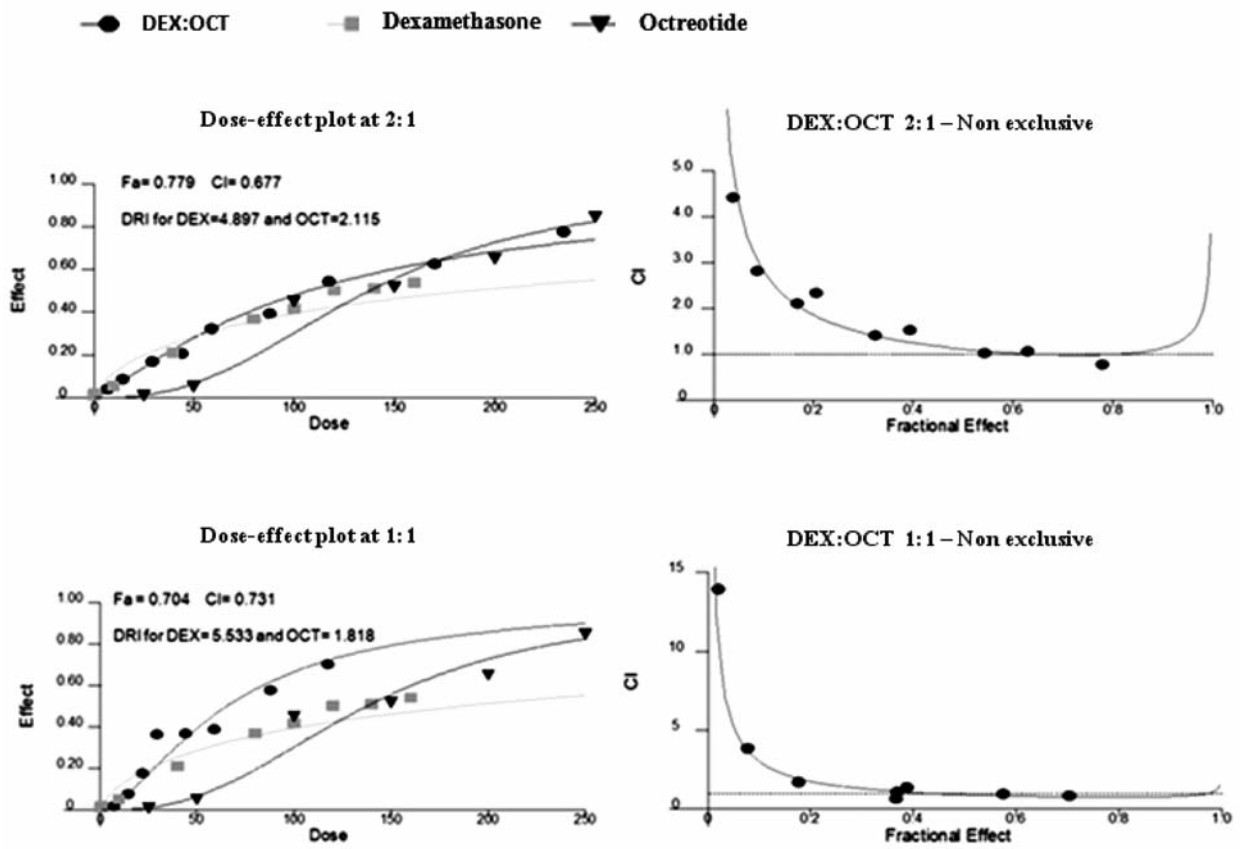

Figure 3. *The anticancer effects of dexamethasone (DEX) and octreotide (OCT) in TRAMP-C1 cells in vitro. The dose-dependent effects and combination index (CI) curves for the various combinations of DEX and OCT on TRAMP-C1 cells (CI <1, =1 and >1) were analyzed as described in the Material and Methods. The dose-reduction index (DRI) represents an estimation of how much the dose of each drug in a synergistic combination can be reduced at a given level of anticancer action as compared with the doses of each drug alone. Our data revealed that the combination of DEX plus OCT is moderately synergistic over a wide range of doses, having CIs of 0.677-0.882 and 0.590-0.820 at 2:1 and 1:1 concentration ratios, respectively.*

lesion grading to C (20%) and B (80%), being the most effective in comparison to any other single treatment scheme. In addition, the combination use of DEX plus OCT produced a good response in bone grading similar to that of DOC [grade B (80%) and grade C (20%)]. The neoadjuvant use of DEX plus OCT prior to DOC treatment further improved the anticancer effectiveness of the single DOC treatment scheme and of the OCT plus DEX combination treatment scheme, reducing bone lesion grading to C (20%), B (60%) and preventing the development of bone lesions in 20% of the inoculated animals (Figure 6B).

Measurements of serum IGF-1, RANKL, OPG and IL-6 in diseased C57Bl mice. Our data showed that the OPG and IL-6 levels were significantly increased and that the IGF-1 and RANKL levels were significantly reduced in the diseased animals (placebo group) as compared to values measured in healthy control C57Bl mice of a similar age (Table III). These data were in concordance with the values expected in animals bearing disseminated disease with bony lesions of blastic nature (increased OPG and reduced RANKL) and being under significant stress (increased IL-6) and remarkable catabolic state (reduced IGF-1 values).

Discussion

The administration of DEX plus SM analog regimen has been reported to confer objective palliative responses in castration resistant prostate cancer patients with diffused bone metastasis (33-38). The clinical responses of a series of phase II clinical studies have documented strong evidence of subjective and objective clinical responses, such as 60% reduction >50% of PSA basal levels within 3 months of treatment initiation, followed by a median progression-free survival of 6-7 months and a median overall survival of 16-18 months (36-38). This novel hormonal manipulation (DEX

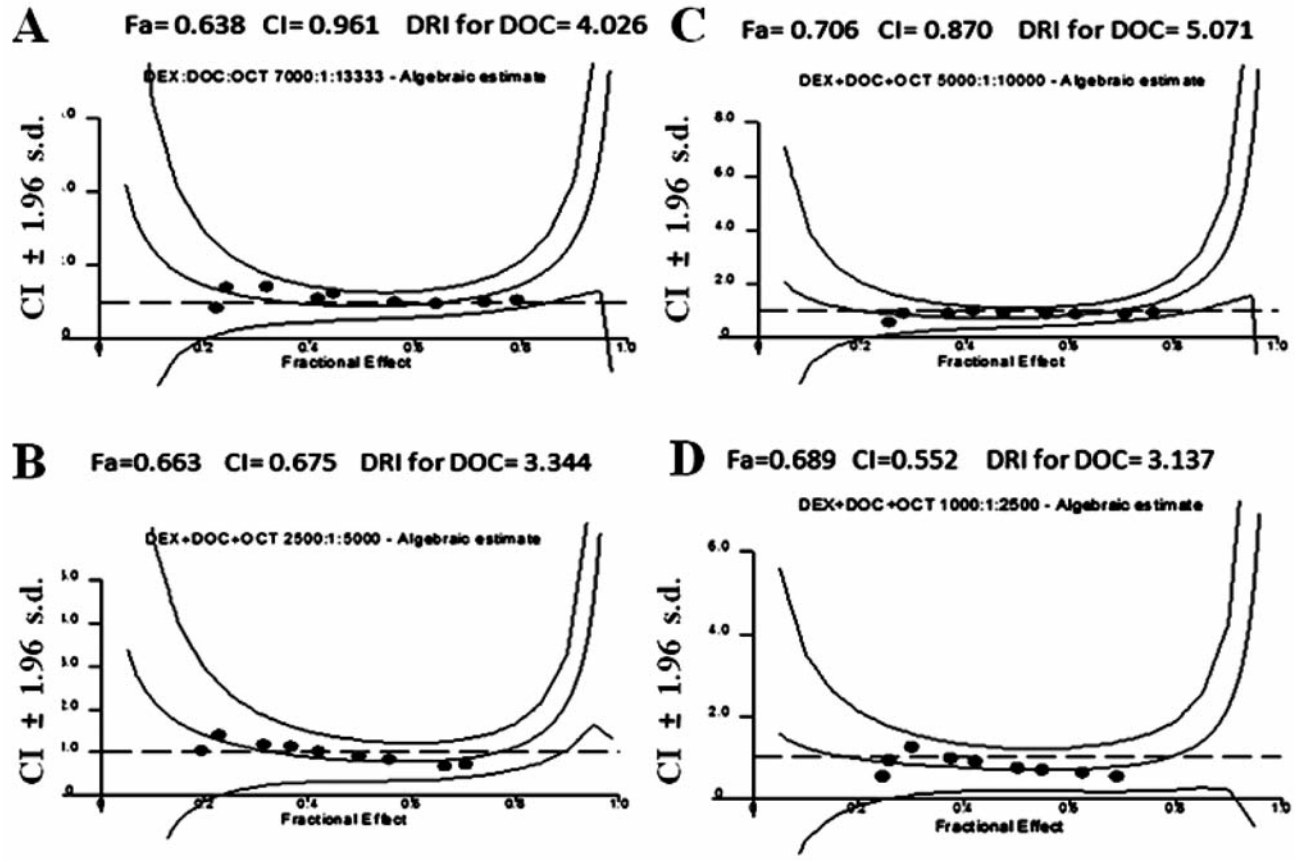

Figure 4. *Analysis of the possible synergistic/additive anticancer effects of dexamenthasone (DEX), octreotide (OCT) and docetaxel (DOC) on TRAMP-C1 cells. Dose effect and combination index (CI) curves for the various combinations with DEX, OCT and DOC on TRAMP-C1 cells. Curves with solid lines (A-D) are computer simulated fa-CI plots for DEX, OCT and DOC. The treatment scheme of DEX plus DOC plus OCT (7000:1:13330, A; 5000:1:10000, B; 2500:1:5000, C; 1000:1:2500, D) produced an additive-synergistic effect (CI: 0.552-0.988) at most ratios tested, and produced strong inhibition of TRAMP-C1 cell growth. A considerable reduction, by 5.071-fold, of the DOC dose necessary to produce maximal inhibition of TRAMP-C1 cells in vitro was detected at 5000:1:10000 concentration ratio.*

plus SM analog regimen) was followed by a significant reduction of IGF-1 serum levels, while clinical responses were not associated with the expression of SSTRs in the bone lesions of responding patients, as assessed by radiolabelled SM analog [^{111}In-DTPA-*D*-Phe^{1}]-octreotide (octreoscan) analysis (36).

Since this hormonal regimen has never been tested in combination with chemotherapy, we designed the present study to try to provide with preclinical evidence for such a therapeutic approach. Thus we used TRAMP-C1 cells as a model of castration-resistant tumor growth inoculating TRAMP-C1 cells onto the femoral periosteum of C57Bl mice. The tumors which developed at the inoculation sites in C57Bl mice did possess a soft tissue component, which produced distant metastasis, mainly in liver and lungs, and a skeletal component presented as a local bony lesion. This bony component of the lesions produced radiographic images, which were easily measurable, enabling the

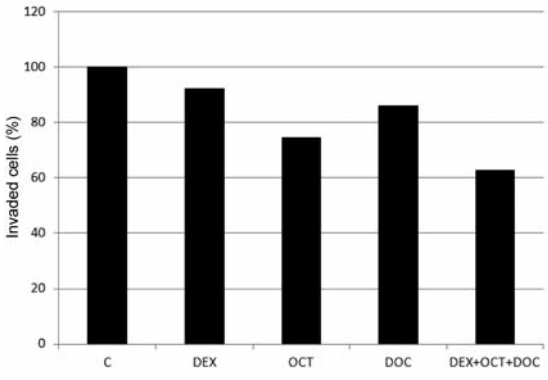

Figure 5. *Analysis of the metastatic potential of TRAMP-C1 cells in vitro, using an invasion assay. Single treatment schemes revealed that the invasive capacity of TRAMP-C1 cells was inhibited by 7.8% using DEX, by 25.5% using OCT and by 4% using DOC. However, the triple combination treatment scheme resulted in an inhibition of 37.3% of the TRAMP-C1 cell invasive potential ($p<0.05$).*

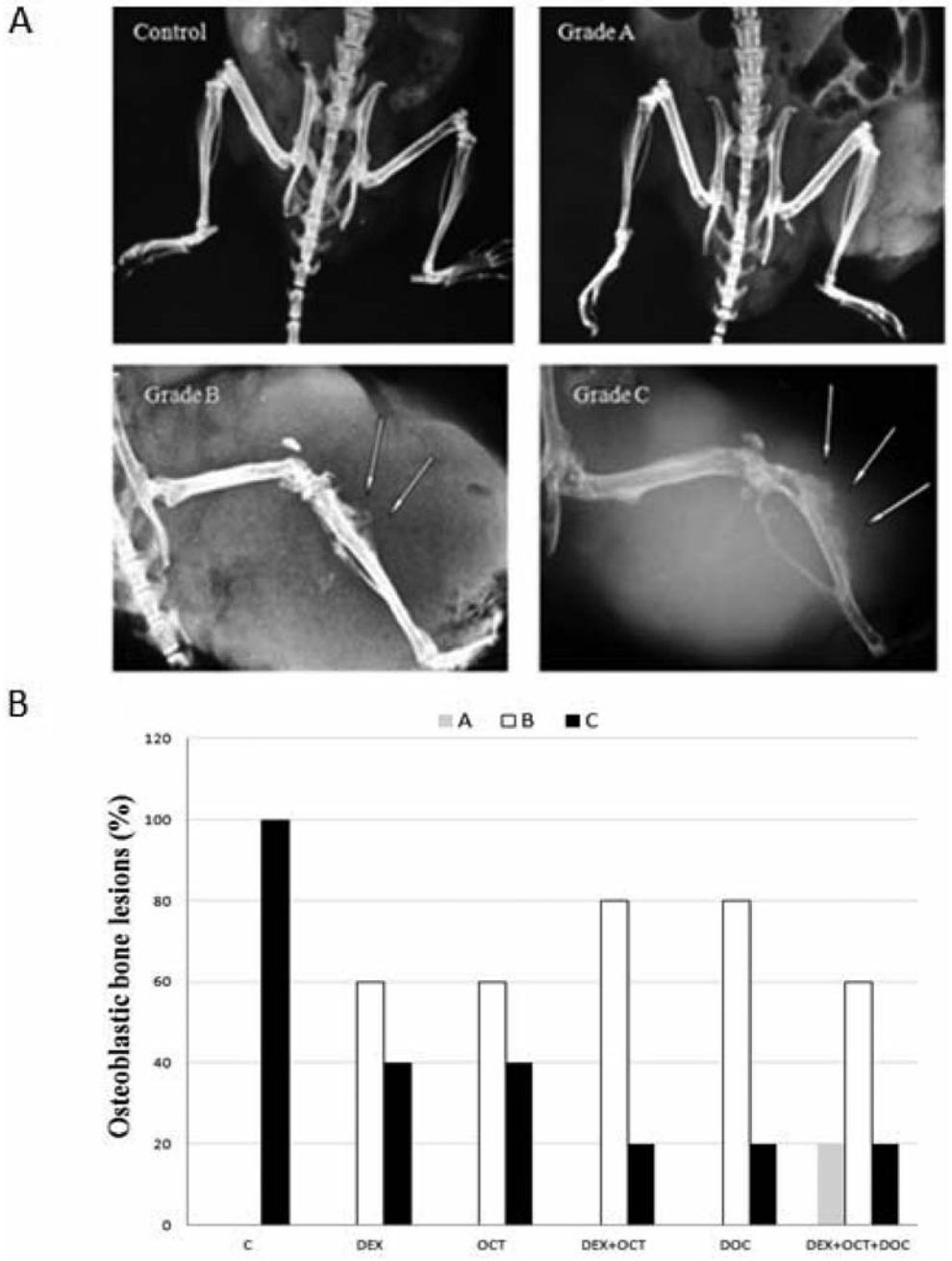

Figure 6. *Analysis of radiographic images of the bone lesions. A: Analysis of the bone lesions based on our grading system using x-ray images of the affected bones, as described under Material and Methods enabled us to assess the anticancer effects of the drugs under investigation. Notably, the bone lesions produced different grades of bone erosion and radiographic evidence of blastic reaction locally. Examples of various grades of bone erosion are presented. The radiographic images were independently evaluated by three experts using a semi-quantitative scoring system categorizing bone lesions as follows: grade A, development of soft tissue tumor with metastasis to distant organs, without affecting the femur; the bone appears normal when compared to the contralateral bone; grade B, development of soft tissue tumor with metastasis to distant organs and presence of bony lesions which are asymmetric and limited to the inoculation site; grade C, significant bony lesions with peripheral bone margin breaks and bone surface disruption. B: Analysis of the grades of the bone lesions detected after the administration of different treatment schemes. The placebo group presented with bony lesions in the inoculation sites of all diseased animals with grade C. Single docetaxel (DOC) treatment scheme produced a significant reduction of bone tumor grading to B and C, which were actually the best anticancer effect on bone lesion as compared to any other single treatment scheme tested. The combination of DEX plus OCT revealed significant reduction of bony tumor grading similar to that of DOC treatment. Notably, the neoadjuvant use of DEX plus OCT prior to DOC treatment scheme reduced bone lesion grading to grade B in the majority of cases and prevented the development of bone lesions in 20% of the inoculated animals.*

Table II. *Antitumor effects of dexamethasone (DEX), octreotide (OCT) and docetaxel (DOC) in single and combination treatment schemes on TRAMP-C1 mouse prostate cancer in vivo.*

Compounds	Treatment schedule[a] (days)	Dose (mg/kg)	MST (days)	T/C (%)	Survivors
DEX	42-49	0.1 *(*s.c.*)	115±3.4	115	0/5
OCT	42	20 (im)	110±8.6	110	0/5
DEX + OCT	42-49 (DEX) 42 (OCT)	0.1 * (*s.c.*) 20 (*i.m.*)	122±9.9	122	0/5
DOC	49	10 (*i.p.*)	133±12.7 $p<0.01$	133	0/5
DEX + OCT + DOC	42-49 (DEX) 42 (OCT) 49 (DOC)	0.1* (*s.c.*) 10 (*i.m.*) 5 (*i.p.*)	150±10 $p<0.01$	150	0/5
Controls	42-49	Saline	100±4.9	100	0/7

Tumor cells were inoculated at day 0. [a]Treatment was started when the tumor was palpable (1 cm^3) approximately 42 days after inoculation. MST=mean survival time (days). T/C=mean survival time (%) of drug-treated animals (T) *versus* saline-treated controls (C). *Dexamethasone dose in mg/day for 7 days.

categorization into a grading scoring system based on the extension of bone destruction. This provided us with the opportunity to assess the anticancer effects of the regimens under investigation on the bony component itself. This bony component appeared of blastic nature, while the diseased animals presented with serum measurements of the bone metabolic markers (IGF-1, IL-6, RANKL and OPG) similar to those measured in the serum of human prostate cancer patients with bone lesions (36-39).

Initially, we characterized the TRAMP-C1 prostate cancer cells for the expression of SSTRs, documenting the expression of SSTR1, SSTR2, SSTR3 and SSTR5. This suggested that the SM analog used in our study (OCT; Sandostatin-LAR; Novartis Pharmaceutical) acts directly on TRAMP-C1-induced tumors in C57Bl mice, apart from any indirect actions exerted on the bone microenvironment-related survival factors, as previously described in the human setting (13-21). This was an important difference *vis-à-vis* the expression analysis of SSTRs in bone lesions detected by radiolabelled SM analog [^{111}In-DTPA-*D*-Phe^1]-octreotide in castration-resistant prostate cancer patients (36). Therefore, the effects of the OCT on TRAMP-C1 models may involve the activation of SSTR2 and SSTR5. These receptors are known to produce cytostasis on several lines of cancer cells (57-59). Recently, SSTR2 activation was also shown to induce apoptosis *via* a p53-independent pathway in cancer cells (60-62).

Indeed, analysis of the anticancer effects of DEX, OCT and DOC in TRAMP-C1 cells has documented the significant inhibitory effects of each of the single agent regimens on TRAMP-C1 proliferation and invasion assays, while providing evidence for their important synergistic effects *in vitro*. In particular, the triple combination treatment scheme reduced the required DOC concentration for maximum inhibition of TRAMP-C1 cell growth by 5-fold. In corroboration with the above, the anticancer effects of such regimens on TRAMP-C1-induced bone lesions in C57Bl mice documented a synergistic effect on the overall survival of these animals. Moreover, the adjuvant use of the DEX plus OCT regimen prior to DOC treatment considerably increased the anticancer effects of DOC in overall survival in this model. Furthermore, the adjuvant use of the DEX plus OCT regimen resulted in the significant reduction of the bone lesion grading, improving the anticancer effects of DOC. Interestingly, this treatment approach inhibited the development of bone tumors in 20% of the inoculated animals. These data suggest that the DEX plus OCT regimen specifically targets the development and growth of the skeletal component of such lesions developed at the inoculation site.

Table III. *Mean (±SD) serum levels of osteoprotegerin (OPG), receptor activator of NF-κβ (RANK) ligand (RANKL), insulin-like growth factor 1 (IGF-1) and interleukin 6 (IL-6) as measured in TRAMP-C1 tumor-bearing mice (placebo group) and healthy C57Bl mice (control group).*

Group	OPG[1] (pg/ml)	RANKL[1] (pg/ml)	RANKL/ OPG	IGF-1[2] (ng/ml)	IL-61 (pg/ml)
TRAMP-C1	4920±30	58±10	0.012±0.003	382±36	89.5±6.7
Healthy C57Bl	1873±68	176±28	0.094±0.017	518±67	7.4±0.64

Previous studies have shown that SM analogs and DEX regimens have a very favourable toxicity profile even at very high doses and offer the advantage of convenient dosing schedules. In addition, the neuroendocrine differentiation of prostate cancer, which is not a very rare event in prostate cancer, is considered to be a crucial component of castration-

resistant prostate cancer growth (40). Therefore, SM analog plus DEX regimen is also expected to have an additional anticancer effect on such histological components of human prostate cancer. Recently, new strategies have been followed in drug design, addressing the possibility of developing new chimeric compounds, also targeting other receptors, such as dopamine receptors (63). These chimeric ligands can exhibit very potent anticancer activities in experimental tumor models, which may be, at least in part, attributed to heterodimerization of the targeted receptors (DA and SSTRs). Moreover, nowadays experimental evidence exists to support the production of cytotoxic SM analogs in conjugation with chemotherapeutic agents which can confer selective binding of such compounds onto SSTR-positive cancer cells, resulting in the accumulation of the chemotherapeutic drug inside the targeted cancer cells. Several such agents have been successfully tested on several experimental models (64-65). The role of SM analogs in the clinical management of the castration-resistant prostate cancer is, therefore, foreseeable. The present study has provided for the first time, pre-clinical data supporting the neoadjuvant use of the DEX plus OCT regimen prior to DOC treatment in a model resembling the clinical setting of castration resistant-prostate cancer.

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