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ORIGINAL ARTICLE

Octreotide in combination with AT-101 induces cytotoxicity and apoptosis through up-regulation of somatostatin receptors 2 and 5 in DU-145 prostate cancer cells

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Abstract Prostate cancer (PCa) is the most common type of cancer among males. Although survival rate of early-stage PCa is high, treatment options are very limited for recurrent disease. In this study, the possible synergistic cytotoxic and apoptotic effect of octreotide in combination with AT-101 was investigated in DU-145 hormone and drug refractory prostate cancer cell line. To enlighten the action mechanisms of the combination treatment, expression levels of somatostatin receptors 2 and 5 (SSTR2 and SSTR5) were also investigated. Cell viability was measured by XTT assay. Apoptosis was assessed through DNA fragmentation analysis and caspase 3/7 assay. mRNA and protein levels of SSTR2 and SSTR5 were evaluated by qRT-PCR and western blot analysis, respectively. Octreotide in combination with AT-101 inhibited cell viability and induced apoptosis synergistically in DU-145 cells as compared to any agent alone. Combination treatment increased both SSTR2 and SSTR5 mRNA and protein levels in DU-145 cells. The data suggest that this combination therapy may be a good candidate for patients with advanced metastatic PCa do not respond to androgen deprivation.

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Introduction

Prostate cancer (PCa) is the most common type of cancer among males [1, 2]. Although survival rate of early-stage PCa is high, treatment options are very limited for recurrent disease. Taxane-based chemotherapy is the first-line option for PCa patients with limited efficacy and serious side effects [2]. Thus, researchers are trying to explore novel cytotoxic agents or combination therapies which sensitize cancer cells to cytotoxicity and apoptosis.

Prostate tissue contains more neuroendocrine cells than other genitourinary system organs. Neuroendocrine cells are androgen receptor and prostate-specific antigen (PSA)-negative and do not proliferate but they regulate growth and differentiation by secreting various neuropeptides [3, 4]. Somatostatin (SST) is the most investigated one among these peptides because of its antineoplastic activity. Anti-proliferative effects of SST and its analogues have been shown in breast, prostate, colon, pancreas, and lung cancer in vitro [5].

Octreotide is one of the synthetic SST analogues which selectively bind to SST receptors, SSTR2 and SSTR5. It is known that these two types of receptors are found in both primary and metastatic hormone-refractory prostatic adenocarcinoma. Inhibition of tumor growth was shown in rat prostate cancer models and in human prostatic cancer cell lines xenografted into nude mice [6, 7]. Clinical studies with metastatic hormone-refractory prostate cancer patients have demonstrated prolonged survival and fall in PSA levels after treatment with some SST analogues [6–8].

AT-101 (R-(-)-gossypol acetic acid) is a polyphenolic compound derived from cotton species, a potent enantiomer of

gossypol [9]. It has been shown that AT-101 has anti-proliferative and apoptotic effects in various cancer tumors in vitro and in vivo. It has been accepted as a novel class of drugs because of its slight side effects as compared to conventional chemotherapeutics and a potential drug for combination therapies [9, 10].

Here, we evaluated the possible synergistic cytotoxic and apoptotic effects of octreotide in combination with AT-101 in DU-145 hormone and drug refractory prostate cancer cell line. Moreover, we investigated both mRNA and protein levels of SSTR2 and SSTR5 after combination treatment.

Materials and methods

Cell culture

Human prostate cancer cell line DU-145 was purchased from Interlab Cell Line Collection (ICLC, Genova, Italy). Cells were cultured in RPMI 1640 medium including 10 % heat-inactivated fetal bovine serum (FBS), 1 %L-glutamine, and 1 % penicillin-streptomycin in 75-cm^2 polystyrene flasks (Corning Life Sciences, UK) and maintained at 37 °C in a humidified atmosphere with 5 % CO_2. AT-101 was as a generous gift from Ascenta Therapeutics (Malvern, PA, USA). Octreotide was obtained from Sigma Chemical Co (St. Louis, MO, USA). All other chemicals were purchased from Sigma Chemical Co (St. Louis, MO, USA).

XTT viability assay

The DU-145 cells were seeded at approximately 1×10^4 cells/well in 96-well flat bottom microtiter plates. To evaluate the effects of octreotide and AT-101 on the viability of prostate cancer cells, DU-145 cells were exposed to increasing concentrations of octreotide (10^{-3}–10^3 nM) and AT-101 (0.25–5 μM) for 24, 48, and 72 h and then cell viability was measured. Treated cells were incubated at 37 °C in a 5 % CO_2 incubator for 24, 48, and 72 h. At the end of incubation period, 100 μl of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (Roche Applied Science, Mannheim, Germany) was added, and plates were incubated at 37 °C for additional 4 h. Absorbance of the wells was measured at 450 nm against a reference wavelength at 650 nm (Beckman Coulter, DTX 880 Multimode Reader, Miami, FL, USA).

DNA fragmentation assay

To investigate the synergistic cytotoxic effect of octreotide/AT-101 combination on apoptotis, we assessed the levels of monooligonucleosome fragments via Cell Death Detection Plus ELISA kit (Roche, Germany). DU-145 cells were treated with octreotide and AT-101 alone or in combination for 72 h. Treated and untreated cells were lysed and centrifuged for 10 min at 200×*g*, and 80 μl of anti-histone-biotin/anti-DNA-POD reagent was added on 20 μl of the supernatant of the lysates and incubated in a streptavidin-coated microplate for 2 h at room temperature. One hundred microliters of substrate solution [2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)] was added to each well, incubated for 20 min, and measured at 405 nm with a reference wavelength at 490 nm (Beckman Coulter, DTX 880 Multimode Reader, Miami, FL, USA).

Caspase 3/7 activity assays

Apoptosis was also verified by using the Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, DU-145 cells at a 10^4 cells/well were plated in a 96-well plate in 100 μl culture medium in the absence or presence of increasing concentrations of drugs alone, or combined, for 72 h. Then 100 μl of Caspase-Glo 3/7 reagent was added on to each well, and the plates were incubated at room temperature for a further 1 h. At the end of the incubation period, the luminescence of each sample was measured by a luminometer (DTX 880 Multimode Reader).

Western blot analysis

To prepare cell lysates for western immunoblot analysis, cell pellets were lysed in buffer containing 20 mM Tris (tris(hydroxymethyl)aminomethane)-HCl (pH 8.0), 137 mM NaCl, 10 % glycerol, 1 % Triton X-100, 1 mM Na3VO4, 25 mM glycerophosphate and phosphatase inhibitor cocktail 1 (Sigma, St. Louis, MO, USA). After centrifugation at 14, 000×*g* for 15 min, protein concentrations were quantitated in duplicate by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were separated on an SDS polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride Immobilon-P membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5 % nonfat dry milk prepared in Tris-buffered saline containing 0.1 % Tween 20 (TBST) at room temperature for 1 h. The membrane was then incubated with primary antibodies (SSTR2, SSTR5, Abcam, UK) at 4 °C overnight. Dilutions of primary antibodies were prepared according to the manufacturer's instructions. Following several washes in TBST, membranes were incubated with appropriate secondary antibodies (1:2000 dilution, Millipore Upstate USA, Charlottesville, VA) at room temperature for 1 h. The protein bands recognized by the antibodies were visualized by the Kodak Gel Logic 1500 Imaging System.

RNA isolation and real-time quantitative PCR (qRT-PCR) assay

The total cellular RNA was extracted from treated and untreated cells via Trizol agent (Qiagen). The cells were lysed in 1 ml

Trizol, incubated at room temperature for 5 min. After incubation period, 200 ml chloroform was added to the lysate, incubated for 3 min more, and centrifuged for 15 min at 12,000×*g* at 4 °C. The aqueous layer was removed, mixed with an equal volume of isopropanol, and incubated for 1 h at 4 °C. The purified RNA was precipitated by centrifugation at 12,000×*g* for 15 min and dissolved in 50 μl diethylpyrocarbonate-treated water. The integrity and quality of the isolated RNA was determined by running the RNAs on agarose gel electrophoresis.

Total RNA (1 μg) was converted to cDNA using the Quantitect reverse transcription kit (Qiagen, USA). qRT-PCR was performed on SSTR2 and SSTR5. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control using a Real-Time TM qPCR Primer Assay (SABioscience, Frederick, MD) on the Light Cycler 480 instrument (Roche Applied Science, Mannheim, Germany). The PCR reaction mixture containing 12.5 μl RT2 SYBR Green qPCR Master Mix, 10.5 μl DNAase-RNase free water, 1.0 μl gene-specific 10 M PCR primer pair stock, and finally 1.0 μl cDNA sample for each primer was prepared at a final volume of 25 μl. Universal cycling conditions (10 min at 95 °C, 15 s at 95 °C, 1 min at 60 °C for 40 cycles) were employed. Data were normalized to the endogenous control GAPDH. Each replicate cycle threshold (CT) was normalized to the average CT of the endogenous control on a sample basis. The comparative CT method was used to calculate the relative quantification of gene expression.

Statistical analysis

All experiments were carried out in triplicate and the results are expressed as the mean±SD, and data was analyzed by using one-way analysis of variance test (ANOVA) followed by Dunnett's *t* test for multiple comparisons. Values with $p<0.05$ were considered as statistically significant. Determination of the half maximal (50 %) inhibitory concentrations (IC_{50}) of each agent and the synergistic cytotoxic effects of the combined treatment of cells with octreotide and AT-101 were calculated by Biosoft CalcuSyn 2.1 software (Ferguson, MO, USA). The combination index (CI) was used to express additive effect (CI =1), antagonism (CI >1), synergism (CI <1), and strong synergism (CI <0.5) [11]. Data analysis and graphs were done by Graphpad Prism 6.0 software (La Jolla, CA, USA).

Results

Octreotide in combination with AT-101 inhibits cell viability synergistically in DU-145 cells

Both octreotide and AT-101 decreased cell viability in a time- and concentration-dependent manner in DU-145 cells (Fig. 1a, b). The highest cytotoxicity was observed at 72 h and IC_{50} values of octreotide and AT-101 in DU-145 cells were found to be 105.6 nM and 3.3 μM, respectively.

To investigate the possible synergistic effects of octreotide and AT-101 combination, DU-145 cells were treated with different concentrations of octreotide and AT-101 alone and in combination for 72 h. Octreotide (0.1 nM) and AT-101 (2.5 μM) resulted in a 23.3 and 47.1 % decrease, respectively, in viability of DU-145 cells, but the combination resulted in a 76.35 % decrease in viability of DU-145 cells ($*p<0.05$) (Fig. 2). The results showed that 0.1 nM octreotide in combination with 0.5 and 1 μM AT-101 resulted in synergistic cytotoxic effect ($*p<0.05$).

We examined the effect of sequential treatment with either octreotide or AT-101 and subsequent treatment with the second agent on DU-145 cells. Pretreatment of DU-145 cells with octreotide for 36 h, a wash, and then treatment for an additional 36 h with AT-101 resulted in synergistic cytotoxicity in DU-145 cells. Pretreatment of cancer cells with AT-101 for

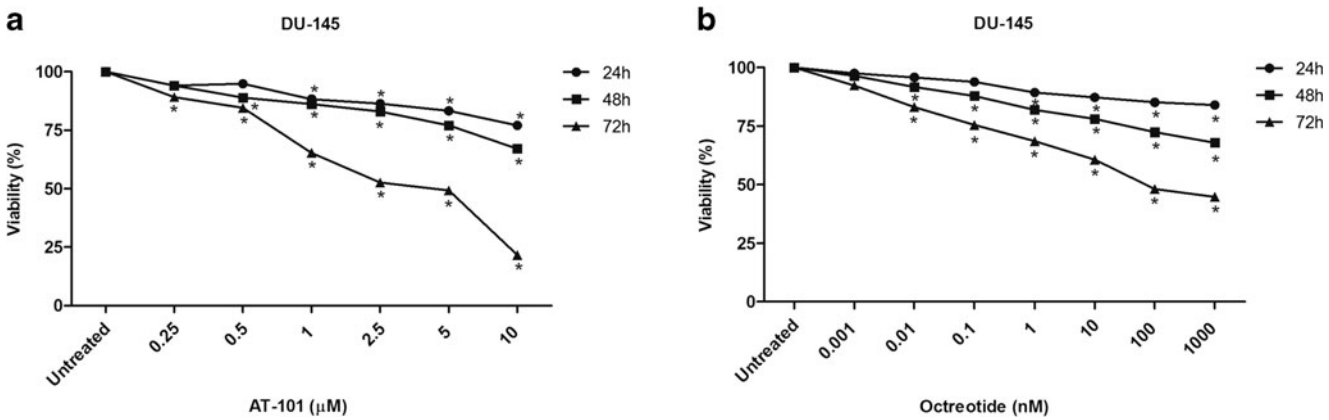

Fig. 1 Time and concentration dependent inhibition of viability by AT-101 (**a**) and octreotide (**b**) in DU-145 cells ($*p<0.05$). The results are expressed as the mean of three different experiments

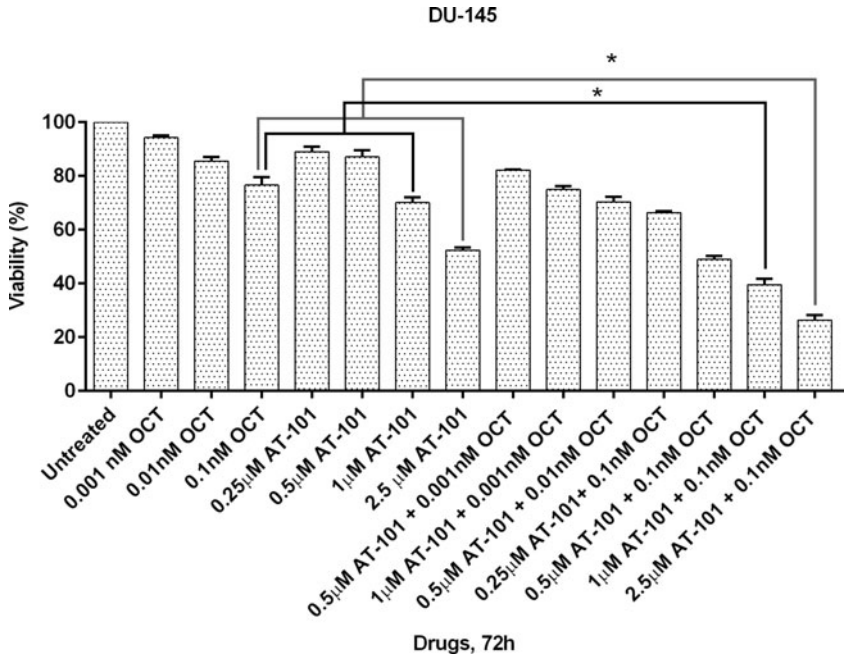

Fig. 2 The effects of AT-101 and octreotide combination on the viability of DU-145 cells at 72 h (*$p<0.05$). The results are expressed as the mean of three different experiments

36 h, a wash, and then treatment for an additional 36 h with octreotide resulted in synergistic cytotoxicity in DU-145 cells (data not shown). Thus, there was a significant synergistic effect of the sequential treatment regardless of which agent is applied first.

Octreotide/AT-101 combination-induced apoptotic cell death in DU-145 cells

To investigate the synergistic cytotoxic effect of octreotide/AT-101 combination on apoptotic cell death, we assessed the levels of monooligonucleosome fragments via Cell Death Detection Plus ELISA kit (Roche, Germany). DU-145 cells were treated with octreotide and AT-101 alone or in combination for 72 h and then DNA fragmentation was analyzed.

When DU-145 cells were exposed to 0.1 nM octreotide and 1 μM AT-101, there was 22.5 and 11.75 %-fold increase in DNA fragmentation, respectively. However, the combination induced 43.75 % increase in DNA fragmentation vs untreated controls (*$p<0.05$) (Fig. 3a). When DU-145 were exposed to 0.1 nM octreotide and 2.5 μM AT-101, there was a 22.5 and 21.75 % increase in DNA fragmentation, and the combination induced an 48.6 % increase in DNA fragmentation vs

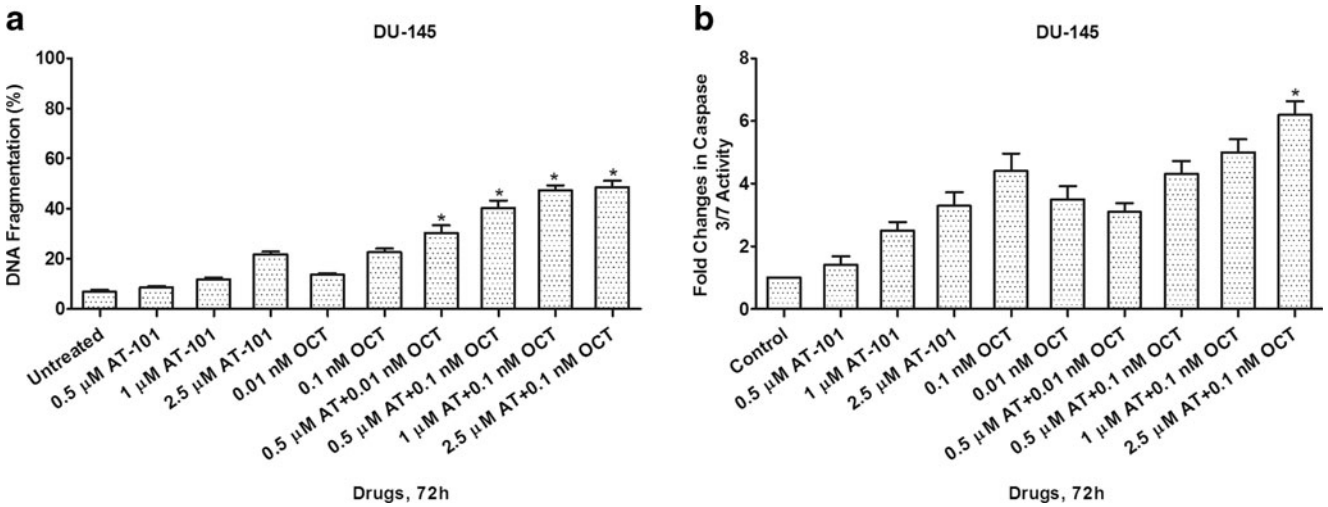

Fig. 3 Apoptotic effects of AT-101 and octreotide in combination on DU-145 cells through DNA fragmentation analyses (**a**) and caspase 3/7 activity (**b**) at 72 h (*$p<0.05$). The results were expressed as the mean of three different experiments

untreated controls indicating synergistic apoptotic effect in prostate cancer cells (*$p<0.05$) (Fig. 3a).

We also verified the apoptosis by caspases 3 and 7 assay. There were 4.4- and 3.3-fold increases in the caspase 3/7 activity between 0.1 nM octreotide and 2.5 μM AT-101 alone-treated DU-145 cells, respectively. However, the combination treatment resulted in a 6.2-fold increase in caspase 3/7 activity (*$p<0.05$) (Fig. 3b).

Combination treatment induced both SSTR2 and SSTR5 expression levels in DU-145 cells

DU-145 cells showed detectable levels of both SSTR2 and SSTR5. However, at the basal level, SSTR2 protein level was higher than the SSTR5 levels in DU-145 cells.

Combination treatment induced both SSTR2 and SSTR5 mRNA and protein levels as compared to any agent alone in DU-145 cells (Fig. 4).

Discussion

Advanced stage PCa becomes hormone refractory after hormonal therapies, and taxanes are the standard chemotherapeutics for these patients [2]. However, progression occurs in most of the patients besides the serious side effects, and developing drug resistance is another important problem in hormone refractory PCa treatment [1, 2]. Therefore, it is important to investigate novel chemotherapeutics or combination therapies for these patients.

AT-101 is one of the mostly investigated agents for hormone refractory PCa. It is demonstrated that it has potent antiproliferative and apoptotic effects in PCa in vitro and in vivo [10, 12–14]. Moreover, combination studies with other chemotherapeutics are very promising indicating that AT-101 may be an effective agent for PCa treatment [13, 15–17].

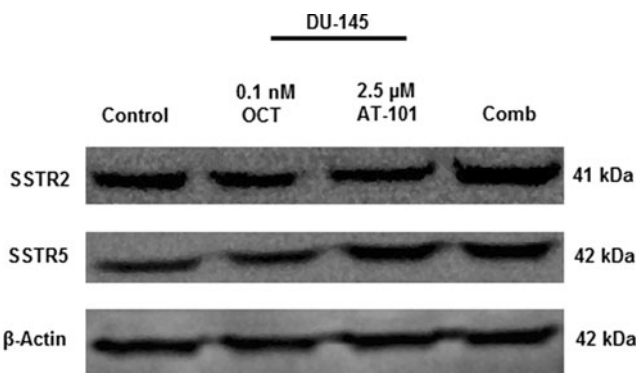

Fig. 4 Western blot analysis of SSTR2 and SSTR5 expressions after AT-101 or octreotide alone and in combination at 72 h (*$p<0.05$). Representative data from several independent experiments are shown

Octreotide is one of the SST analogues which extensively studied for its anti-tumor properties. After detection of SST receptors in malign tissues, anti-proliferative effects of octreotide were shown in breast, prostate, colon, pancreas, and lung cancer [8].

There are many studies demonstrating the synergistic effects of AT-101 in combination with various agents in lung, breast, and ovarian cancer. However, this is the first study demonstrating the synergistic effect of AT-101 in combination with octreotide in prostate cancer cells.

Further, we investigated the apoptotic effect of the combination treatment in DU-145 cells. Results showed that octreotide in combination with AT-101 induced DNA fragmentation as compared to single agents alone. In our previous studies, we have shown that single octreotide treatment induces apoptosis in prostate cancer cell lines DU-145 and PC-3. However, up to our knowledge, this is the first study investigating the apoptotic effect of AT-101 in combination with octreotide in prostate cancer cells. Since activation of apoptosis is a favorable feature for potential chemotherapeutics, octreotide in combination with AT-101 may be a good option for hormone refractory PCa patients because of its strong synergistic apoptotic activity.

Further, we tried to enlighten the underlying mechanisms of synergistic effect of the combination treatment in DU-145 cells. Since most of the octreotide analogues, including octreotide, bind to SSTR2 and SSTR5, we investigated the levels of these two receptors in both mRNA and protein levels. qPCR results showed that SSTR2 and SSTR5 mRNA levels were up-regulated in combination treated DU-145 cells as compared to single agents. Similarly, protein levels of both receptors were also induced in DU-145 cells. Up-regulation of SSTR2 and SSTR5 in DU-145 cells by the combination treatment may be one of the underlying mechanisms of the octreotide/AT-101 combination. Although it is known that SST receptors are found in normal tissues, clinical studies with radiolabeled SST analogues in cancer patients have shown very low toxicity in pituitary, kidney, or bone marrow tissues but significant reduction in tumor growth. Thus, our results should be extended to normal prostate cancer cells as well as other types of prostate cancer cells in future studies.

This study indicates that combined treatment of octreotide and AT-101 in DU-145 cells resulted in a greater inhibition of cancer cell proliferation and significantly induced apoptosis and SST 2/5 receptor levels. Our findings suggest that this combination therapy may be a good candidate for patients with advanced metastatic prostate cancer do not respond to androgen deprivation.

Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

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