Inhibition of Neurotensin Receptor 1 Selectively Sensitizes Prostate Cancer to Ionizing Radiation

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Abstract

Radiotherapy combined with androgen depletion is generally successful for treating locally advanced prostate cancer. However, radioresistance that contributes to recurrence remains a major therapeutic problem in many patients. In this study, we define the high-affinity neurotensin receptor 1 (NTR1) as a tractable new molecular target to radiosensitize prostate cancers. The selective NTR1 antagonist SR48692 sensitized prostate cancer cells in a dose- and time-dependent manner, increasing apoptotic cell death and decreasing clonogenic survival. The observed cancer selectivity for combinations of SR48692 and radiation reflected differential expression of NTR1, which is highly expressed in prostate cancer cells but not in normal prostate epithelial cells. Radiosensitization was not affected by androgen dependence or androgen receptor expression status. NTR1 inhibition in cancer cell–attenuated epidermal growth factor receptor activation and downstream signaling, whether induced by neurotensin or ionizing radiation, establish a molecular mechanism for sensitization. Most notably, SR48692 efficiently radiosensitized PC-3M orthotopic human tumor xenografts in mice, and significantly reduced tumor burden. Taken together, our findings offer preclinical proof of concept for targeting the NTR1 receptor as a strategy to improve efficacy and outcomes of prostate cancer treatments using radiotherapy. Cancer Res; 71(21); 1–10. ©2011 AACR.

Introduction

Prostate cancer is the most common cancer in men and the second leading cause of cancer deaths in the United States (1,2). Radiotherapy is one of the standard treatment modalities for prostate cancer (3); however, a major obstacle to effective radiotherapy is the limited radiation dose that can be safely delivered to the prostate (<85 Gy; refs. 4, 5). Unfortunately, at this dose level, a significant proportion of tumors are resistant either not responding or recurring after treatment. An alternative to radiation dose increase would be to use radiosensitizing agents selectively targeted to prostate cancer cells while sparing normal tissue, thus minimizing radiation toxicity by lowering effective therapeutic doses.

Several different factors could participate in prostate cancer development, progression, and resistance to antitumor therapy. One of such possible mechanisms involves intraprostate neuroendocrine cells and their secretions, which can aid cancer cell proliferation and survival. Neuroendocrine cells exist in the normal prostate gland, regulating prostatic growth, differentiation, and secretion. However, clusters of neuroendocrine-like cells are also found in most prostate cancer, and the presence of extensive neuroendocrine features in tumors is an indication of increased aggressiveness and androgen independence (6–8). These neuroendocrine-like cells often arise from prostate cancer cells through the process of neuroendocrine transdifferentiation (7, 9). The neuroendocrine-like cells secrete a variety of factors, including parathyroid hormone–related peptides, serotonin, calcitonin, bombesin-related peptide, and neurotensin, that enhance DNA synthesis, proliferation, and migration of prostate cancer cells in vitro. In vivo, neuroendocrine-like cells can promote androgen-independent LNCaP xenograft growth in castrated mice (10, 11), supporting their role in prostate cancer androgen-independent growth.

One of the neuropeptides secreted by neuroendocrine-like prostate cells is neurotensin (12), a 13 amino acid peptide, that has numerous physiologic effects (13) mediated predominantly through its cognate high-affinity receptor, neurotensin receptor 1 (NTR1). Similar to other G-protein–coupled receptors, stimulated NTR1 activates multiple pathways, namely, mobilization of intracellular Ca\(^{2+}\), production of cyclic AMP and GMP, and formation of inositol triphosphate, resulting in important physiologic responses in both the central nervous system and periphery. However, it has been shown that neurotensin has significant stimulatory activity in several human neoplastic tissues (13–15). For example, NTR1 is...
expressed in 91% of invasive ductal breast cancer cases (16), and its expression is associated with the grade and size of the tumor and its invasive potential (17). Similarly, NTR1 is expressed in most colon cancer cell lines and primary tumors, especially in the highly invasive adenocarcinomas but not in normal adult colon cells (18). In addition, neurotensin and NTR1 expression has been implicated in the invasiveness of head and neck squamous cell carcinomas, and high levels of NTR1 expression are a predictive marker for a poor prognosis (19). We and others have shown that NTR1 is expressed and activated in aggressive prostate cancer cells (15, 20, 21) but not in normal prostate epithelial cells. In such prostate cancer cells, stimulation with neurotensin increased mitogen–activated protein kinase and phosphoinositide-3-kinase activation (22) and epidermal growth factor receptor (EGFR), Src, and STAT5 phosphorylation (15, 22), resulting in enhanced DNA synthesis, cell proliferation, and survival.

NTR1 signaling may not only be responsible for increased proliferation but also for the intrinsic radiosensitivity of prostate cancer cells. Thus, inhibition of the NTR1 receptor and its downstream signaling represents a target to enhance the sensitivity of prostate cancer to radiotherapy. NTR1 can be selectively and efficiently inhibited by the commercially available small-molecule antagonist, SR48692 (medicinent; Sanofi-Aventis; refs. 23–25). SR48692 is a nonpeptide antagonist that binds preferentially to NTR1 and inhibits downstream signaling events such as EGFR and Src activation. SR48692 has been shown in vitro to be effective in inhibiting proproliferative and prosurvival signaling in colon (26), pancreatic (27, 28), head and neck (19), and prostate cancer cells (15, 29). SR48692 has also shown promising activity in vivo in a small lung cancer mouse model (30). However, its potential use as a radiosensitizer has never been tested.

In the current study, we tested the hypothesis that inhibition of NTR1 by SR48692 would radiosensitize prostate cancer cells and tumors. Our results show that, indeed, combined treatment of SR48692 and radiation effectively kills cancer cells in vitro and in vivo and suggest the need for clinical testing to establish whether anti-NTR1 treatment combined with radiotherapy may increase local control of the tumor and decrease its metastatic potential.

Materials and Methods

Cell lines and reagents

PC-3M-luc-C6 (PC-3M) human prostate carcinoma cell line transfected with the luciferase gene was purchased from Xenogen Corporation, which also provided luciferin. RWPE-1 (immortalized normal prostate epithelial cells) cell line was obtained from American Type Culture Collection. These 2 cell lines were authenticated by the suppliers, and fresh-frozen stocks were used for the experiments. The LNCaP (androgen-dependent human prostate carcinoma) and C4-2B cell lines (androgen-independent human prostate carcinoma) were established and characterized by Dr. L. W. Chung at the University of Virginia (31) and resuscitated from frozen stocks prior to the experiments. All cell lines were maintained in a 37°C/5% CO2 humidified chamber in Dulbecco’s Modified Eagle’s Media supplemented with 10% FBS (PC-3M), RPMI supplemented with 5% FBS (LNCaP and C4-2B), or serum-free keratinocyte medium supplemented with EGF and bovine pituitary extract (RWPE-1). Phenol red–free and serum-free RPMI medium was used in all serum starvation experiments. All cell culture media and reagents were purchased from Invitrogen. SR48692, a generous gift from Sanofi-Aventis, was dissolved in dimethyl formamide (DMF) as a 2 mmol/L stock and stored at −80°C. Antibodies specific for EGFR, EGFR phospho-tyrosine 992 and 845, and Src and Src phospho-tyrosine 416 were obtained from Cell Signaling Technology, pan-phospho-tyrosine (Y20) from Abcam, and secondary antibodies conjugated to infrared dyes from LI-COR. SDS-PAGE electrophoresis, agarose gel electrophoresis supplies, and PCR reagents were obtained from BioRad. SMARTpool NTR1 and nontargeting siRNA were obtained from Dharmacon. Other miscellaneous reagents were purchased from Sigma Chemicals.

Drug treatment and irradiation

Incubation with SR48692 was conducted at 37°C at 1 μmol/L and 24 hours before irradiation, except where noted. An equivalent volume of drug vehicle (DMF) was added to the control dishes (final concentration ≤ 0.01%). Drug-treated and control cells were irradiated using a 220-keV X-ray irradiator at a dose rate of 2 to 3 Gy/min and received a dose of 6 Gy, except where noted. All irradiations were carried out at room temperature, and the samples were returned to 37°C (for further incubation) or lysed on ice (to assess immediate effects).

Clonogenic survival assay

The assays were conducted as described previously (32). In brief, exponentially growing cells were treated with SR48692 (or sham treated with vehicle) and irradiated. Cells were trypsinized, rinsed, and counted, and appropriate numbers were plated for the colony formation assay. After 10 to 14 days of incubation, colonies consisting of more than 50 cells were counted. All data points were determined in triplicate and experiments were carried out at least 3 times. The experimental results were fitted to standard linear quadratic dose–response curves and corrected for effects induced by the drug treatment alone. The dose enhancement ratio (DER) was defined as the ratio between the radiation dose in the absence of radiosensitizer and the dose in the presence of radiosensitizer resulting in the same reduction of cell survival to 37% (DER57).

Growth inhibition assay

Cells (100,000 cells per well) were plated in 6-wells plates, allowed to attach, and treated with SR48692 for indicated times. Attached cells were collected at indicated time points and counted using trypan blue exclusion to discriminate live/dead cells, or the cell number was estimated by sulforhodamine B staining of cellular proteins.

Cell lysates, immunoprecipitation, and Western blotting

Cells were plated in 100-mm dishes (2 × 10⁶ cells per dish), allowed to attach overnight, and treated with SR48692
(or sham treated) as indicated in the Results section. Following irradiation (6 Gy) and different postincubation times, cells were washed with ice-cold PBS and harvested on ice in CHAPS lysis buffer (0.6% CHAPS, 1% Triton X-100, 50 mmol/L Tris, pH = 8.0, 150 mmol/L NaCl, 2 mmol/L EDTA) supplemented with protease and phosphatase inhibitors (Sigma; ref. 15). Lysates were clarified by centrifugation, and protein concentrations were quantified. To assess whole-cell protein expression, equal amounts of protein were resolved on 4% to 15% gradient gels, transferred to nitrocellulose, and probed with specific antibodies. Infrared dye–conjugated secondary antibodies were used for signal development, and quantification of signal intensity was conducted using a LiCOR Odyssey imager and software. For immunoprecipitation, 1 mg of precleared protein lysate was incubated overnight at 4°C with EGFR antibody (5 μg; Cell Signaling) or a negative control antibody, with constant rocking. Protein A/G agarose beads were then added, and immunoprecipitates were washed 3 times with lysis buffer. Finally, samples were eluted with 2× sample buffer (100°C/5 minutes) and subjected to Western blotting procedure as described above.

RNA isolation and reverse transcription PCR

Total cellular RNA was isolated from exponentially growing cells using the RNeasy Kit (Qiagen). Reverse transcription was carried out with 2 μg of total RNA using iScript reagent (Bio-Rad). The PCR amplification was carried out using 25 pmol of specific NTR1, NTR2, NTR3, and neurotensin primers as described elsewhere (ref. 33; NTR1, forward 5'-TCTCGGCTTGGTGGCTGCT-3', reverse 5'-TGTTGCTGGACACGCTGTCG-5'; NTR2, forward 5'-GCTCCAGCTCATCGTAT-3', reverse 5'-TCCCAAAAGCCTGAAGCTGTA-3'; NTR3, forward 5'-AGAATGGTCAGACATGTGT-3', reverse 5'-AAGACTTATCCAAGGAGTCC-3'; and neurotensin, forward 5'-ACTTGCTTGTGAGAACGGG-3', reverse 5'-TTGGACGCTGTATATTGTCC-3') and JumpStart REDTaq polymerase mix (Sigma). The thermal profile consisted of initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 50 seconds, extension at 72°C for 45 seconds, and by a final extension at 72°C for 7 minutes. PCR products were resolved on a 1% agarose gel, stained with SYBR Safe, and quantified using the ImageJ Program (http://rsweb.nih.gov/ij/). The NTR3-specific product was used to normalize the signals.

Caspase activation assay

Cells were plated in 96-well plates (10,000 cells per well), allowed to attach, treated with SR48692 (1 μmol/L) or sham treated (equal volume of DMF) for 24 hours, and irradiated (6 Gy). Following 24 to 48 hours of incubation, the activation of cellular caspases 3 and 7 was measured by a fluorescence-based assay (Apo-ONE® Homogeneous Caspase-3/7 Assay; Promega). Multiple (6–12) wells per treatment condition were used to obtain average fluorescence signals and normalized to sham-treated controls.

In vivo orthotopic human prostate cancer xenografts

Human PC-3M-luc-C6 cells were used in an orthotopic xenograft model in male athymic nude mice [NIH Balb/cAn NCr-nu (nu/nu); 5–6 weeks old]. Surgery was conducted to expose the prostate, and 20 μl of the tumor cell suspension (5 × 106 cells) was injected into the dorsolateral lobe of the prostate gland. Following closure, mice were treated with SR48692 (25 mg/kg in PEG800) or vehicle control for 5 consecutive days, according to the schedule described in the Results section. Drug was administered orally in 0.2 mL volumes 4 hours prior to radiation treatments. Mice were then anesthetized with a ketamine/xylazine mix, and a dose of 2.5 Gy X-ray was given to the prostate area while shielding the body with lead (34). Animals were imaged on a weekly basis by anesthetizing with isoflurane before and during imaging and injecting intraperitoneally with luciferin (a substrate for luciferase) at 150 mg/kg in a volume of 0.1 mL (35). Animals were imaged at a peak time of 10 to 20 minutes post-luciferin injection via a Xenogen IVIS instrument, using exposure times and sensitivity settings to avoid saturation. Image processing was conducted using Living Image software (Xenogen) by region-of-interest analysis of total photons/sec for each tumor, with appropriate background subtraction.

Statistical analysis

All error bars represent the SEM from n = 3 independent experiments, unless indicated otherwise. P values were calculated using a one-factor ANOVA; values of P < 0.05 were considered statistically significant.

Results

SR48692 sensitizes PC-3M prostate cancer cells to ionizing radiation

We tested the hypothesis that inhibiting the NTR1 receptor, and therefore its downstream proproliferation and prosurvival signals, will enhance cell-killing effects of ionizing radiation in prostate cancer cells. For initial experiments, we chose an androgen-independent and highly metastatic prostate adenocarcinoma cell line (PC-3M) and a nontumorigenic prostate epithelial cell line (RWPE-1). Cells were treated with an NTR1 antagonist, SR48692, irradiated, and their colony-forming (clonogenic) ability assessed. At this concentration, SR48692 significantly (P < 0.01) enhanced radiation effects in PC-3M cells (Fig. 1A), resulting in a decrease in surviving fraction at 2 Gy (SF2) from 0.575 to 0.331, and a clinically relevant dose enhancement ratio at 37% survival (DER37) of 1.77. As shown in Supplementary Fig. S1, SR48692-induced radiosensitization is dose and time dependent, reaching a maximum at 1 μmol/L and 24 hours of treatment. Importantly, SR48692 pretreatment did not sensitize normal epithelial RWPE-1 cells (Fig. 1B).

Prostate cancer cells, but not normal prostate epithelial cells, express NTR1

The results of Fig. 1A and B showed that SR48692-induced radiosensitization is specific to prostate cancer cells. Here, we confirmed that neurotensin receptors (NTR1, NTR2, and NTR3) are differentially expressed in normal and cancer
prostate cell lines. Fig. 1C shows that NTR1 protein, the specific target of SR48692, is expressed in PC-3M cells but not in RWPE-1 cells. Surprisingly, the mRNA for NTR1 was present in both cell lines (Fig. 1D), although the level in RWPE-1 was approximately 50% lower than in PC-3M cells (Fig. 1E). Both cell lines contained similar levels of mRNA for NTR3 (Fig. 1D, NTR3 lanes) but none for NTR2 (Fig. 1D, NTR2 lanes). Protein levels of NTR3 and NTR2 correlated with mRNA levels (data not shown). NTR1 protein was also expressed in LNCaP, C4-2B, and DU-145 prostate cancer cells at comparable levels (Supplementary Fig. S3). In addition, PC-3M expressed mRNA for the neurotensin/neuromedin gene, whereas the neurotensin-specific reverse transcription PCR (RT-PCR) product was absent in RWPE-1 (Fig. 1D, neurotensin lanes). This finding was confirmed using an ELISA assay (Supplementary Fig. S4), where we detected significant levels of neurotensin in PC-3M cell culture medium but not in RWPE-1 or LNCaP culture media. These observations suggest that autocrine stimulation of prostate cancer cells occurs via endogenously expressed agonist (neurotensin), which stimulates expressed NTR1.

To validate our hypothesis that the radiosensitizing effects of SR48692 are mediated through its interactions with NTR1, we silenced the receptor mRNA expression in PC-3M cells using SmartPool siRNA (Dharmacon). The results in Supplementary Fig. S2 show that knocking down of NTR1 radiosensitized prostate cancer cells (Supplementary Fig. S2B) and reduced cell proliferation (Supplementary Fig. S2A). Importantly, treatment with SR48692 did not further increase the radiosensitivity of PC-3M cells with depleted NTR1 receptor.

**SR48692 alone inhibits neurotensin-induced prostate cancer cell growth**

Existing literature suggests that NTR1 stimulation enhances prostate cancer cell growth, and inhibiting this pathway with SR48692 reduces cell proliferation. Our results (Fig. 1) show that SR48692 reduces clonogenic survival of cancer cells exposed to ionizing radiation; however, our preliminary experiments suggested that the drug alone did not significantly affect cell survival under similar conditions. Thus, we attempted to reconcile these observations using PC-3M cells maintained under different conditions: exponentially growing cells in complete medium (as used in radiosensitization experiments) and quiescent cells in serum-free medium (as used in stimulation/proliferation experiments; refs. 15, 22). As shown in Fig. 2A, neurotensin stimulated proliferation of serum-starved PC-3M cells, whereas blocking the NTR1 receptor with SR48692 (concomitant with neurotensin stimulation) completely abrogated this response. Moreover, SR48692 pretreatment diminished neurotensin-induced EGFR and Src phosphorylation in

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**Figure 1.** Blocking NTR1 receptor sensitizes prostate cancer cells to ionizing radiation. A, clonogenic survival of PC-3M cells treated with SR48692 and radiation. Cells were incubated with 1 μmol/L SR48692 (SR) for 24 hours or left untreated (Ctrl), irradiated and replated for colony formation. Results are normalized for effects of the drug alone and fitted to a standard linear quadratic model. Data points are derived from at least 4 independent experiments (SEM). **, P < 0.01 denotes statistical significance compared with radiation only. B, clonogenic survival of RWPE-1 normal prostate epithelial cells treated with SR48692 and radiation. C, expression of NTR1 receptor protein in RWPE-1 and PC-3M cell lines assessed by Western blotting. D, expression of neurotensin receptors (NTR1, NTR2, and NTR3) and neurotensin (NT) mRNA in RWPE-1 and PC-3M cell lines assessed by semiquantitative RT-PCR. E, quantification of RT-PCR products from agarose gel electrophoresis (D). Expression of NTR3 was used to normalize signal intensities.
serum-starved PC-3M cells (Fig. 2B). RWPE-1 normal prostate epithelial cells did not respond to neurotensin stimulation nor SR48692 treatment(s) (Fig. 2A and C) most likely because they do not possess NTR1 receptors (Fig. 1C and D).

Strikingly, PC-3M cells grown in complete medium were minimally affected by SR48692 treatment. Fig. 2D shows that SR48692 minimally affected cell growth, and similar negligible effects were noted in long-term clonogenic survival assays (14 days colony formation). Even at the highest dose tested (10 μmol/L), the drug effects did not exceed 30% inhibition. In agreement with these findings, SR48692 had no effect on cell-cycle progression of PC-3M cells growing exponentially in complete medium (Fig. 2E). On the basis of these results, we can also conclude that observed radiosensitizing activity in cells growing in complete medium is not due to effects such as cell-cycle perturbation and/or induction of cell death by the drug alone.

**SR48692 radiosensitizes androgen-sensitive cell lines LNCaP and C4-2B**

To determine whether SR48692-radiosensitizing activity is confined only to androgen-insensitive PC-3M cells or is also

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**Figure 2.** Differential effects of NTR1 antagonist in serum-free versus serum-containing medium. A, SR48692 inhibits stimulatory effects of neurotensin in prostate cancer cells maintained in serum-free medium. PC-3M and RWPE-1 cells were serum starved for 48 hours, pretreated with SR48692 (SR; 1 μmol/L for 24 hours), stimulated with neurotensin (NT; 100 nmol/L for 24 hours) and proteins quantified by sulforhodamine B staining. Results are normalized to the untreated control, and data points are from 2 to 4 independent experiments (SEM). B, neurotensin-induced EGFR and Src phosphorylation in serum-starved PC-3M cells. Cells were lysed following 15 to 60 minutes of incubation with neurotensin (100 nmol/L) with or without SR48692 (1 μmol/L), cellular EGFR was immunoprecipitated (IP), and EGFR phosphorylation was assessed using phosphospecific antibodies (tyrosine 845, pY845; tyrosine 992, pY992). Src phosphorylation (tyrosine 416, pY416) was assessed using straight Western blotting (WB) of protein lysates. Total EGFR and Src levels were also assessed. C, under similar conditions (serum-free medium), neurotensin (100 nmol/L for 1 hour) does not induce EGFR phosphorylation in RWPE-1 cells lacking NTR1 receptor. EGF stimulation (100 ng/mL for 5 minutes) was used as a positive control. D, NTR1 antagonist has a minimal effect on growth and survival of prostate cancer cells maintained in complete growth medium. PC-3M cells were treated with indicated concentrations of SR48692 for 24 hours and counted (growth) or maintained continuously for 14 days. Established colonies scored (survival). E, SR48692 has no effect on cell-cycle progression in cells maintained in serum-containing medium. Exponentially growing PC-3M cells were treated with SR48692 (SR; 1 μmol/L for 24 hours), and the cell-cycle distribution was assessed by BrdUrd incorporation and DNA staining. *, P < 0.05; **, P < 0.01 denote statistical significance compared to control samples.
present in androgen-sensitive prostate cancer cells, we tested LNCaP and its derivative, C4-2B, in clonogenic survival assays. Both cell lines express androgen receptor and respond to androgen stimulation, unlike PC-3M, which is androgen receptor negative and androgen insensitive. The difference between the two cell lines is that C4-2B can grow in the absence of androgen, whereas LNCaP growth is androgen dependent. In addition to androgen receptor status, LNCaP and C4-2B cell lines also differ significantly from PC-3M in their p53 status: Both express wild-type protein, whereas PC-3M cells are p53 null. As shown in Fig. 3A and B, both cell lines were sensitized to radiation by pretreatment with SR48692. The decreases in SF$_2$ (LNCaP from 0.599 to 0.419, C4-2B from 0.481 to 0.336) following combined treatment are statistically significant ($P < 0.05$), although smaller in magnitude than the decrease in SF$_2$ in PC-3M cells, whereas the DER$_{27}$ (1.43) calculated for both cell lines is also smaller than for PC-3M cells. On the basis of these observations, we hypothesize that inhibition of NTR1 radiosensitizes prostate cancer cells independently of their androgen receptor and p53 status.

**SR48692 affects EGFR phosphorylation in response to radiation or neurotensin**

It is known that radiation induces EGFR phosphorylation and activates downstream signaling pathways leading to increased cell survival (36, 37). Therefore, we determined whether SR48692 pretreatment interferes with radiation- and/or neurotensin-induced EGFR phosphorylation. For short-term studies in complete medium, the SR48692-treated PC-3M cells were irradiated, incubated for 5 minutes at 37°C, and lysed in CHAPS buffer. Total cellular EGFR was immunoprecipitated, and Western blotting was carried out using antibodies against EGFR phosphotyrosine 992 (pY992) and total phosphotyrosine (pY20). Fig. 4A shows that under these conditions, radiation induced an increase in EGFR phosphorylation as determined by both pY20 and pY992 phospho-specific antibodies. SR48692 alone did not affect EGFR phosphorylation levels; however, it reduced radiation-induced phosphorylation to 83% (pY992) and 67% (pY20) of initial levels.

Fig. 4B shows a modest increase in the phosphorylation levels of EGFR (pY845 and pY992) and Src (pY416) in serum-starved PC-3M cells following an extended time course (15–60 minutes) of irradiation alone (X) and significant increase with combined treatment of radiation plus neurotensin (X + NT). The radiation-induced phosphorylation, observed 5 minutes postirradiation in Fig. 4A, was reduced to almost basal levels at 15 or 60 minutes following irradiation (Fig. 4B, X). With combined treatment (Fig. 4B, X + NT), EGFR and Src phosphorylation reached a maximum at 60 minutes, closely
Combined treatment of SR48692 with radiation elicits synergistic antitumor activity against PC-3M tumor xenografts in mice

The orthotopic PC-3M human prostate cancer model was used to test the hypothesis that SR48692 can be used as a radiosensitizing agent in vivo. The schematic outlines of the in vivo experiments are presented in Fig. 6A and the results in Fig. 6B and C. In the first experiment, animals (8 per treatment group) were randomized on day 5 postinoculation, and drug treatment/irradiation was started immediately thereafter. In the second experiment, tumor development was monitored by bioluminescence for 14 to 21 days, and then, the animals with similar tumor burden were randomized into treatment groups and treated. Treatment regimen was the same in both experiments and consisted of 5 daily doses of SR48692 (25 mg/kg) followed by 2.5 Gy ionizing radiation on days 3 and 5 (Fig. 6A).

Fig. 6B and C shows the tumor burden (measured as bioluminescence from PC-3M cells) in animals in experiments 1 and 2, respectively. In both experiments, SR48692 alone (SR) had no significant effect on tumor progression, whereas radiation alone (X) was only partially efficacious. However, the combined treatment (X + SR) showed the most prominent effect, significantly reducing tumor growth in the treated animals (X vs. X + SR; P < 0.05). This reduction was especially noticeable when the combined treatment was used on animals inoculated only 5 days before (Fig. 6B, experiment 1). The reduced tumor burden was confirmed by visual examination of excised urogenital tracts (Fig. 6D, representative samples from experiment 2).

Discussion

On the basis of the incidence of cancer recurrence and radioresistance, improvements in radiation therapy, in general, and in prostate tumor treatments, in particular, are urgently needed. In the present study, we show that inhibition of NTR1 is a novel method for radiosensitization of prostate cancer. Our results show that combined treatment of SR48692, a selective inhibitor of NTR1, and ionizing radiation efficiently kills cancer cells in vitro and significantly lowers tumor burden in vivo. In addition, combination of NTR1 antagonist and radiotherapy is effective independent of cancer cell p53 and androgen receptor status. Most importantly, the combined treatment provides selectivity between normal and cancer cells.

The magnitude of sensitization to radiation seen here with SR48692 in cell lines (Figs. 1 and 3, DER17 ~ 1.3–1.8) is lower than often observed with anticancer drug sensitization. Nevertheless, the differential radiosensitization is significant as compared with, for example, clinically used and experimental EGFR targeting sensitizing agents such as erlotinib (40) or C225 (41, 42). Our in vivo experiments confirm that SR48692 treatment combined with radiation causes significant reduction in tumor growth (Fig. 6).

We postulate that the differential expression of NTR1, its high expression in prostate cancer cells but not in normal prostate epithelial cells (Fig. 1), is a major factor in the selectivity of SR48692-induced radiosensitization. However,
there are at least 3 different neurotensin receptors (NTR1, NTR2, and NTR3; ref. 43), therefore, one may ask whether SR48692-induced radiosensitization is related to the inhibition of NTR2 and/or NTR3. In support of our hypothesis, SR48692 has been shown to have a high selectivity for NTR1 and does not interact strongly with NTR2 or NTR3 (44, 45) or other unrelated receptors (44). In addition, the literature (33) and our results (Fig. 1D) show that NTR2 is not expressed in most prostate cells (including cell lines used in this study). The case of NTR3 is more complicated—this receptor is ubiquitously expressed throughout the human body and highly expressed in the studied cell lines (Fig. 1D). However, its activation is not likely blocked by SR48692 treatments, as there are 2 orders of magnitude difference in $K_d$ for binding to NTR1 and NTR3 (45, 46). NTR3 has been shown to cooperate with NTR1 in transducing the neurotensin signal (33, 45). Therefore, it is not very probable, although still possible, that SR-induced radiosensitization is a result of blocking neurotensin binding/stimulation of NTR3 receptor. However, the requirement of NTR1 in SR48692-induced radiosensitivity was confirmed using siRNA-mediated silencing of the receptor (Supplementary Fig. S2). On the basis of these considerations, we believe that the observed radiosensitizing effect is due predominantly to the inhibition of NTR1.

In addition to the differences in NTR1 expression, the differences in neurotensin secretion could contribute to cancer selectivity of SR48692 combined with radiation. Our results (Fig. 1D and Supplementary Fig. S4B) show that PC-3M cancer cells express and secrete neurotensin, whereas normal prostate cells do not. The autocrine loop in cells expressing both the receptor (NTR1) and its agonist (neurotensin) could significantly enhance survival after irradiation, and disruption with SR48692 should result in radiosensitivity. It has been reported that abdominal irradiation increases neurotensin secretion in rat ileum (47). Although we did not observe similar effects in cell culture, it is plausible that it can occur in vivo during prostate radiotherapy.

Several possible mechanisms of NTR1-dependent radiosensitization could be operative, including, but not limited to, the disruption of cell-cycle progression, inhibition of DNA damage signaling and/or repair, and enhancement of apoptotic responses. Our results (Fig. 2B) clearly show that SR48692 has no effect on cell cycle of cancer cells growing in complete medium, whereas under these conditions, radiosensitization is observed. This rules out the cell-cycle perturbation as a major factor in sensitization. Even though SR48692 has only a minimal effect on PC-3M cell growth in complete medium, it can completely block neurotensin-induced EGFR and Src phosphorylation (Fig. 4A) and significantly lower radiation-induced EGFR phosphorylation (Fig. 4A). It is known that neurotensin functions as a prosurvival and mitogenic stimulus in cancer cells, operating mostly through EGF receptor…

Figure 6. Effects of NTR1 antagonist combined with radiation on the growth of PC-3M orthotopic xenografts in nude mice. A, schematic description of experiments 1 and 2 as detailed in the Results section. B, experiment 1: 5 days postinoculation mice were randomized, treated with SR48692 (SR; 25 mg/kg) for 5 consecutive days, and irradiated (X; 2.5 Gy delivered to prostate area only) on second and fourth days of treatment. Bioluminescence imaging was conducted on a weekly basis. Data points represent means from 8 animals in the group (SEM). *, $P < 0.05$ compared with radiation only (one-factor ANOVA). C, experiment 2: the presence and size of tumors were assessed 2 weeks postinoculation, and the mice were randomized into 4 treatment groups. Treatment was conducted during week 4 (as described for A). D, representative images (from C) of the urogenital system in nontreated (normal), PC-3M inoculated (Ctrl), and treated (X and X + SR) mice. Samples were collected from mice euthanized 50 to 70 days postgrafting.
transactivation (15, 29). It has also been shown that AKT acts downstream of EGFR (48) as an important prosurvival and antiapoptotic factor in response to ionizing radiation (49, 50). Reducing the threshold to undergo apoptosis by interference with apoptosis resistance pathways would be expected to sensitize tumor cells to ionizing radiation by conditioning them to induction of cell death. Our results support the hypothesis that NTR1 inhibition lowers, or completely abrogates, EGFR phosphorylation (Fig. 4), which results in an increase in apoptosis following irradiation (Fig. 5). Interestingly, it has been reported that neurotensin acts as an antiapoptotic factor, protecting serum-starved breast cancer cells from apoptosis in vitro (51). It is possible that a similar mechanism is involved in prostate cancer cell resistance to radiation-induced apoptosis and that blocking NTR1 abrogates the effects of neurotensin. Additional studies are underway to delineate the molecular mechanism(s) responsible for the induction of apoptosis by treatment with SR48692 combined with radiation.

Previous reports have shown that SR48692 inhibits DNA synthesis and proliferation in serum-starved (quiescent) and neurotensin-stimulated PC-3 cells (15, 22). We have confirmed it here and showed that the drug has a minimal effect on cancer cells growing in complete medium with 10% FBS (Fig. 2). Only when combined with ionizing radiation, SR48692 activity became apparent and biologically significant. These results could also explain the failure of SR48692 in a single-agent clinical trial in patients with lung cancer. Although SR48692 has been shown to inhibit proliferation in vitro and tumor growth in vivo of small-cell lung cancer (30), it did not show significant activity in the clinical trial (NCT00299095). Our observation could also provide a biological basis for an additional mode of cancer-selective radiosensitization in clinical practice. A systemic treatment with nontoxic doses of SR48692, together with spatially targeted delivery of radiotherapy to the particular tumor site, could result in minimal side effects and sparing of the surrounding normal tissue. This, combined with the specificity afforded by the differences in NTR1 expression between normal and cancer cells, would significantly improve the therapeutic index of prostate tumor radiotherapy.

Recently, several groups have reported on the involvement of NTR1 receptor expression and neurotensin stimulation in the proliferation of various cancer cell lines and aggressiveness of tumors in humans (13, 14, 16–19, 21, 26–28, 30). We hypothesize that the molecular mechanism of cancer-selective radiosensitization induced by SR48692 could be similar in different cell/cancer types and mostly based on differential NTR1 expression and its connection to prosurvival and antiapoptotic pathways through EGFR and mitogen-activated protein kinase signaling. Therefore, a combined therapy, as proposed here for prostate tumors, should be applicable to other cancers that express NTR1 receptor.

In summary, we have showed that SR48692 radiosensitizes prostate cancer cells in vitro, enhances radiation-induced apoptosis, and that a decrease in EGFR phosphorylation and signaling may be involved in this effect. As a whole, the data suggest the existence of a cross-talk between EGFR and NTR1 and, possibly, a method for prostate cancer to modulate responses to radiotherapy. Most importantly, the in vivo administration of SR48692 prior to radiotherapy resulted in a significantly improved tumor response compared with the individual agents in human prostate cancer orthotopic xenografts. The results presented here propose that targeting NTR1 receptor with a selective antagonist, such as SR48692, represents a promising new therapeutic strategy for radiosensitization of prostate tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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