Inhibition of neurotensin receptor 1 selectively sensitizes prostate cancer to ionizing radiation†

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† Dedicated to the memory of Dr. George P. Amorino.

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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 cells attenuated EGFR activation and downstream signaling, whether induced by neurotensin or ionizing radiation, establish a molecular mechanism for sensitization. Most notably, SR48692 efficiently sensitized PC-3M orthotopic human tumor xenografts in mice, significantly reducing 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 employing radiotherapy.
INTRODUCTION

Prostate cancer (PCa) 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 PCa (3), however, a major obstacle to effective radiotherapy is the limited radiation dose that can be safely delivered to the prostate (less than 85 Gy) (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 PCa cells while sparing normal tissue, thus minimizing radiation toxicity by lowering effective therapeutic doses.

Several different factors could participate in PCa development, progression, and resistance to antitumor therapy. One of such possible mechanisms involves intra-prostate neuroendocrine cells and their secretions, which can aid cancer cell proliferation and survival. Neuroendocrine (NE) cells exist in the normal prostate gland, regulating prostatic growth, differentiation and secretion. However, clusters of NE-like cells are also found in most PCas, and the presence of extensive NE features in tumors is an indication of increased aggressiveness and androgen independence (6-8). These NE-like cells often arise from PCa cells through the process of neuroendocrine trans-differentiation (7,9). The NE-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 PCa cells in vitro. In vivo, NE-like cells can promote androgen-independent LNCaP xenograft growth in castrated mice (10,11), supporting their role in PCa androgen-independent growth.

One of the neuropeptides secreted by NE-like prostate cells is neurotensin (NT)(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 physiological responses in both the central nervous system and periphery. However, it has been shown that NT 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, NT and NTR1 expression have been implicated in invasiveness of head and neck squamous cell carcinomas, and high levels of NTR1 expression is 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 PCa cells, stimulation with NT increased MAP and PI3 kinase activation (22) and 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 for the intrinsic radioresistance of PCa 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 (Meclnertant, Sanofi-Aventis) (23-25). SR48692 is a non-peptide 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 pro-proliferative and pro-survival signaling in colon (26), pancreatic (27,28), head and neck (19), and PCa cells (15,29). SR48692 has also shown promising activity \textit{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 \textit{in vitro} and \textit{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 (Alameda, CA), which also provided luciferin. RWPE-1 (immortalized normal prostate epithelial cells) cell line was obtained from American Type Tissue Collection (ATTC; Rockville, MD). These two 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 line (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: DMEM 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 (Carlsbad, CA). SR48692, a generous gift from Sanofi-Aventis (Malvern, PA), was dissolved in dimethyl formamide (DMF) as a 2 mM stock and stored at -80°C. Antibodies specific for EGFR, EGFR phospho-tyrosine 992 and 845, Src and Src phospho-tyrosine 416 were obtained from Cell Signaling Technology (Beverly, MA), pan-phospho-tyrosine (Y20) from Abcam (Cambridge, MA), and secondary antibodies conjugated to IR dyes from LiCOR (Lincoln, NE). SDS-PAGE electrophoresis, agarose gel electrophoresis supplies and PCR reagents were obtained from BioRad (Hercules, CA). SMARTpool NTR1 and non-targeting siRNA were obtained from Dhharmacon (Lafayette, CO). Other miscellaneous reagents were purchased from Sigma Chemicals (St. Louis, MO).

**Drug treatment and irradiation**

Incubation with SR48692 was conducted at 37 °C at 1 uM 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 2-3 Gy/min and received a dose of 6 Gy, except where noted. All irradiations were performed 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 and rinsed, counted, and appropriate numbers were plated for the colony
formation assay. After 10 to 14 days of incubation, colonies consisting of >50 cells were counted. All data points were determined in triplicate and experiments were conducted at least three 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% (DER\textsubscript{37}).

**Growth inhibition assay**

Cells (100,000 cells/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 sulforodamine B staining of cellular proteins.

**Cell lysates, immunoprecipitation and Western blotting**

Cells were plated in 100-mm dishes (2 x10\textsuperscript{6} cells/dish), allowed to attach overnight and treated with SR48692 (or sham-treated) as indicated in the Results sections. Following irradiation (6 Gy) and different post-incubation times, cells were washed with ice-cold PBS and harvested on ice in CHAPS lysis buffer (0.6% CHAPS, 1% Triton X-100, 50 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA) supplemented with protease and phosphatase inhibitors (Sigma) (15). Lysates were clarified by centrifugation, and protein concentrations were quantified. To assess whole-cell protein expression, equal amounts of protein were resolved on 4-15% gradient gels, transferred to nitrocellulose and probed with specific antibodies. IR-dye conjugated secondary antibodies were used for signal development, and quantification of signal intensity was performed 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 μl, IP-specific mAb, Cell Signaling) or a negative control antibody, with constant rocking. Protein A/G agarose beads were then added, and immunoprecipitates were washed three times with lysis buffer. Finally, samples were eluted with 2x sample buffer (100 ºC/5 min) and subjected to Western blotting procedure as described above.

**RNA isolation and reverse transcription PCR (RT-PCR)**

Total cellular RNA was isolated from exponentially growing cells using the RNAeasy kit (Qiagen). Reverse transcription was performed 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 (NT) primers as described elsewhere (33) (NTR1, forward 5'-TCATCGCCTTTGTGGTCTGCT-3', reverse 5'-TGGTTGCTGGACACGCTGTCG-3'; NTR2, forward 5'-GTCTCCTCAGCTTCATCGTAT-3', reverse 5'-TCCCCAAAGCCTGAAGCTGTA-3'; NTR3, forward 5'-AGAATGGTGCAGACTATGTTG-3', reverse 5'-AAGAGCTATTCCAAGAGGTCC-3'; NT, forward 5'-ACTTGGCTTGTTAGAAGGC-3', reverse 5'-TGTGGAGCTGGTATATTGTC-3') and JumpStart REDTaq polymerase mix (Sigma). The thermal profile consisted of initial denaturation at 95 ºC for 5 min, followed by 30 cycles of denaturation at 94 ºC for 30 sec, annealing at 62 ºC for 60 sec, extension at 72 ºC for 45 sec, and by a final extension at 72 ºC for 7 minutes. PCR products were resolved on a 1% agarose gel, stained with SYBR Safe and quantified using the ImageJ program (http://rsbweb.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 (1μM) or sham-treated (equal volume of DMF) for 24h, and irradiated (6 Gy).
Following 24-48h incubation, the activation of cellular caspases 3 and 7 was measured by a fluorescence-based assay (Apo-ONE® Homogeneous Caspase-3/7 Assay, Promega, Madison, WI). 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 performed to expose the prostate, and 20 μl of the tumor cell suspension (5 x 10⁵ 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 five consecutive days, according to the schedule described in the Results section. Drug was administered orally in 0.2 ml volumes 4 h 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 (i.p.) 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 10-20 min post-luciferin injection via a Xenogen IVIS instrument, using exposure times and sensitivity settings to avoid saturation. Image processing was performed using Living Image software (Xenogen) by region-of-interest (ROI) analysis of total photons/sec for each tumor, with appropriate background subtraction.

**Statistical analysis**

All error bars represent the standard error of the mean from n = 3 independent experiments, unless indicated otherwise. P values were calculated using a one-factor ANOVA;
p values less than 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 pro-proliferation and pro-survival 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 non-tumorigenic 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 (SF$_2$) from 0.575 to 0.331, and a clinically-relevant dose enhancement ratio at 37% survival (DER$_{37}$) of 1.77. As demonstrated in Supplemental Fig. S1, SR48692-induced radiosensitization is dose- and time-dependent, reaching a maximum at 1 μM and 24h 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 neurotensin receptor 1**

The results of Fig. 1A and B demonstrated that SR48692-induced radiosensitization is specific to PCa cells. Here, we confirmed that neurotensin receptors (NTR1, NTR2 and NTR3), are differentially expressed between normal and cancer prostate cell lines. Figure 1C shows that NTR1 protein, the specific target of SR48692, is expressed in PC-3M cells but not in RWPE-1 cells. Surprisingly, the messenger RNA for NTR1 was present in both cell lines.
(Fig. 1D), although the level in RWPE-1 was ~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 is also expressed in LNCaP, C4-2B and DU-145 prostate cancer cells at comparable levels (supplemental Fig. 3). In addition, PC-3M expressed mRNA for the neurotensin/ neuromedin gene, while the neurotensin-specific RT-PCR product was absent in RWPE-1 (Fig. 1D, NT lanes). This finding was confirmed using an ELISA assay for NT levels in cell culture medium (supplemental Fig. 4), where we detected significant levels of NT 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 exists via endogenously expressed agonist (NT) stimulating 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 supplemental Figure 2 demonstrate that knock-down of NTR1 radiosensitizes PCa cells (Fig. S2B) and reduces cell proliferation (Fig. S2A). Importantly, treatment with SR48692 does not increase radiosensitivity of PC-3M cells with depleted NTR1 receptor.

**SR48692 alone inhibits neurotensin-induced PCa cell growth**

Existing literature suggests that NTR1 stimulation enhances PCa cell growth, and inhibiting this pathway with SR48692 reduces cell proliferation. Our results (Fig. 1) demonstrate that SR48692 reduces clonogenic survival of cancer cells exposed to ionizing radiation; however, our preliminary experiments suggested that the drug alone does not significantly affect cell survival under similar conditions. Here, 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 (15,22)). As shown in Fig. 2A, neurotensin (NT) stimulates proliferation of serum-starved PC-3M cells, while blocking the NTR1 receptor with SR48692 (concomitant with NT-stimulation) completely abrogates this response. Moreover, SR48692 pretreatment diminishes NT-induced EGFR and Src phosphorylation in serum-starved PC-3M cells (Fig. 2B). RWPE-1 normal prostate epithelial cells do not respond to neurotensin stimulation nor SR48692 treatment(s) (Fig. 2A and 2C) most likely because they do not possess NTR1 receptors (Fig. 1C/D).

Strikingly, PC-3M cells grown in complete medium are minimally affected by SR48692 treatment. Fig. 2D demonstrates that SR48692 minimally affects 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 μM), 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). Based on 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 if SR48692 radiosensitizing activity is confined only to androgen-insensitive PC-3M cells or also is present in androgen-sensitive PCa 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 AR-negative and
androgen-insensitive. The difference between the two cell lines is that C4-2B can grow in the absence of androgen, while LNCaP growth is androgen-dependent. In addition to androgen receptor status, LNCaP and C4-2B cell lines also differ significantly from PC-3M by their p53 status: both express wild-type protein, while PC-3M cells are p53-null. As demonstrated in Figure 3A and B, both cell lines are 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, while the DER$_{37}$ calculated for both cell lines (1.43) is also smaller than for PC-3M cells. Based on these observations, we hypothesize that inhibition of NTR1 radiosensitizes prostate cancer cells independently of their AR 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). Here, we determined if SR48692 pretreatment interferes with radiation- and/or NT-induced EGFR phosphorylation. For short-term studies in complete medium, the SR48692-treated PC-3M cells were irradiated, incubated for 5 min at 37 °C, and lysed in CHAPS buffer. Total cellular EGFR was immunoprecipitated, and Western blotting was performed using antibodies against EGFR phosphotyrosine 992 (pY992) and total phosphotyrosine (pY20). Figure 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.
Figure 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 min) of irradiation alone (X) and significant increase with combined treatment of radiation plus neurotensin (X+NT). The radiation-induced phosphorylation, observed 5 min post-irradiation in Fig. 4A, is reduced to almost basal levels at 15 or 60 min following irradiation (Fig. 4B, X). With combined treatment (Fig. 4B, X+NT), EGFR and Src phosphorylation reaches a maximum at 60 min, closely following the kinetics of phosphorylation induced by NT alone (Fig. 2B). In both cases, pretreatment with SR48692 completely abrogate this long-term EGFR or Src phosphorylation (Fig. 4B, SR lanes).

**Pretreatment with SR48692 enhances radiation-induced apoptosis in PC-3M cells**

Treatment of PC-3M cells with a combination of SR48692 and radiation resulted in a significant increase in apoptosis within 48 hours, as shown by the enhanced cleavage of PARP and phospho-S2065 DNA-PK (Fig. 5A, lower bands). Both proteins are known to be targets for caspase 3-mediated degradation during apoptosis (38,39). Consistent with this observation, there was a statistically significant (p<0.05) increase in caspase 3 and 7 activity, as measured with specific fluorescent substrate in cells treated with combined agents (Fig. 5B, X+SR) when compared to radiation only treatment (Fig.5B, X). It is worth to note that, at the dose and times assessed, the drug alone in the presence of serum did not induce cellular apoptotic death, in line with our previous results on the effects of SR48692 on cell growth and cell cycle (Fig. 2D and E).

**Combined treatment of SR48692 with radiation elicits synergistic antitumor activity against PC-3M tumor xenografts in mice**

The orthotopic PC-3M human PCa 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 post-inoculation, and drug treatment / irradiation was started immediately thereafter. In the second experiment, tumor development was monitored by bioluminescence for 14-21 days, 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 five daily doses of SR48692 (25 mg/kg) followed by 2.5 Gy IR on days 3 and 5 (Fig. 6A).

Figure 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, while 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 five days before (experiment 1, Fig. 6B). The reduced tumor burden was confirmed by visual examination of excised urogenital tracts (Fig. 6D, representative samples from experiment 2).

DISCUSSION

Based on 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 demonstrate that inhibition of neurotensin receptor 1 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 \textit{in vitro} and significantly lowers tumor burden \textit{in vivo}. Additionally, 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 recorded here with SR48692 in cell lines (Figs 1 and 3, DER$_{37}$ ~ 1.3-1.8) is lower than often observed with anti-cancer 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 \textit{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 PCa cells but not in normal prostate epithelial cells (Fig. 1), is the major factor in the selectively of SR48692-induced radiosensitization. However, there are at least three different neurotensin receptors (NTR1, NTR2, and NTR3 (43)); therefore, one could ask if 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). Additionally, 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 two orders of magnitude difference in $K_d$ for binding to NTR1 and
NTR3 (45,46). NTR3 has been shown to cooperate with NTR1 in transduction of NT stimulation signals (33,45). Therefore, it is not very probable, although still possible, that SR-induced radiosensitization is a result of blocking NT binding/stimulation of NTR3 receptor. However, the requirement of NTR1 in SR48692-induced radiosensitivity was confirmed using siRNA-mediated silencing of the receptor (Fig. S2). Based on these considerations, we believe that the observed radiosensitizing effect is due mainly to the inhibition of NTR1.

In addition to the differences in NTR1 expression, the differences in NT secretion could contribute to cancer-selectivity of SR48692 combined with radiation. Our results (Fig. 1D and Fig. S4B) show that PC-3M cancer cells express and secrete NT, while normal prostate cells do not. The autocrine loop in cells expressing both the receptor (NTR1) and its agonist (NT) could significantly enhance survival after irradiation, and disruption with SR48692 should result in radiosensitivity. It has been reported that abdominal irradiation increases NT 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 demonstrate that SR48692 has no effect on cell cycle of cancer cells growing in complete medium, while 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 NT-induced EGFR and Src phosphorylation (Fig. 4A) and significantly lower radiation-induced EGFR phosphorylation (Fig. 4A). It is known that NT functions as a pro-survival and mitogenic stimulus in cancer cells, operating mostly through EGFR receptor transactivation (15,29). It
has also been demonstrated that AKT acts downstream of EGFR (48) as an important pro-survival and anti-apoptotic 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 anti-apoptotic factor, protecting serum-starved breast cancer cells from apoptosis in vitro (51). It is possible that a similar mechanism is involved in PCa cell resistance to radiation-induced apoptosis and that blocking NTR1 abrogates the effects of NT. 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 NT-stimulated PC-3 cells (15,22). We have confirmed it here and demonstrated 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 lung cancer patients. Although SR48692 has been shown to inhibit proliferation in vitro and tumor growth in vivo of small cell lung cancer (30), it did not demonstrate significant activity in the trial clinical (NCT0029095). Our observation could also provide a biological basis for an additional mode of cancer-selective radiosensitization in clinical practice. A systemic treatment with non-toxic 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 NT-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 pro-survival and anti-apoptotic pathways through EGFR and MAP 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 demonstrated that SR48692 radiosensitizes prostate cancer cells in vitro, enhancing 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 to 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.

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FIGURE LEGENDS

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 μM SR48692 for 24h (SR) or left untreated (Ctrl), irradiated and re-plated 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 four independent experiments (±SEM); *, ** denote statistical significance at p < 0.05 and <0.01, respectively, compared to 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, 2, 3) and neurotensin (NT) mRNA in RWPE-1 and PC-3M cell lines assessed by semi-quantitative RT-PCR. E) Quantification of RT-PCR products from agarose gel electrophoresis (Panel D). Expression of NTR3 was used to normalize signal intensities.

Figure 2. Differential effects of NTR1 antagonist in serum-free vs. 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 48h, pretreated with SR48692 (SR, 1 μM for 24h), stimulated with neurotensin (NT, 100 nM for 24h) and proteins quantified by sulphorodamine B staining. Results are normalized to the untreated control, and data points are from 2-4 independent experiments (±SEM). B) NT-induced EGFR and Src phosphorylation in serum-starved PC-3M cells. Cells were lysed following 15 to 60 min incubation with NT (100 nM) +/- SR48692 (1 μM), cellular EGFR was immunoprecipitated, and EGFR phosphorylation was assessed using phospho-specific antibodies (tyrosine 845, pY845, and tyrosine 992, pY992). Src phosphorylation (tyrosine 416,
pY416) was assessed using straight Western blotting of protein lysates. Total EGFR and Src levels were also assessed. C) Under similar conditions (serum-free medium), NT (100 nM/1h) does not induce EGFR phosphorylation in RWPE-1 cells lacking NTR1 receptor. EGF stimulation (100 ng/mL for 5 min) 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 24h 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 μM for 24h), and the cell cycle distribution was assessed by BrdU incorporation and DNA staining.

**Figure 3. Inhibition of NTR1 radiosensitizes androgen receptor expressing and androgen-sensitive prostate cancer cells.** LNCaP (A) or C4-2B (B) PCa cells were pre-treated with SR48692 (SR, 1μM for 24h) or sham-treated (Ctrl), irradiated and subjected to a colony formation assay. Data were gathered from at least three independent experiments (±SEM); * and ** denote statistical significance at p < 0.05 and <0.01, respectively, compared to radiation only.

**Figure 4. SR48692 affects neurotensin- and radiation-induced EGFR phosphorylation.**
A) PC-3M prostate cancer cells were treated with SR48692 (SR, 1 μM for 24h) and/or irradiation (X, 6 Gy), and cellular lysates were prepared after 5 min. Total tyrosine (pY20) and EGFR tyrosine 992 specific (pY992) phosphorylation were assessed in EGFR immunoprecipitates. Fold-changes represent the mean of two independent experiments, with phospho-specific signal intensities normalized to total EGFR levels. B) Serum-starved PC-3M
cells were pre-incubated with SR48692 (SR, 1 μM/24h), stimulated with neurotensin (NT, 100 nM/1h) as indicated and irradiated (X, 6 Gy). EGFR phosphorylation (Y845 and Y992), Src phosphorylation (Y416), and the expression of total EGFR and Src proteins were analyzed by immunoprecipitation and Western blotting.

**Figure 5. SR48692 treatment enhances apoptosis in irradiated prostate cancer cells. A)** PC-3M cells were treated with SR48692 (SR, 1 μM for 24h) and irradiated (X, 6 Gy), and samples including floating cells were collected at specified times (24, 48 and 72h post-irradiation). A representative Western blot shows full size and cleaved PARP and phosphorylated (pS2056) DNA-PK after indicated treatments. β-actin was used as a loading control. **B)** Caspase 3/7 activity in SR48692-treated (SR, 1 μM for 24h) and irradiated (X, 6 Gy) PC-3M cells was measured 24 and 48 hours post-irradiation as described in Materials and Methods. Fluorescence signals were normalized to the fluorescence of sham-treated controls (Ctrl). The results were obtained in two independent experiments (6-12 intra-experimental replicates), * indicate p<0.05.

**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 – five days post-inoculation mice were randomized, treated with SR48692 (SR, 25 mg/kg) for five consecutive days and irradiated (X, 2.5 Gy delivered to prostate area only) on second and fourth day of treatment. Bioluminescence imaging was performed on a weekly basis. Data points represent means from 8 animals in the group (±SEM); *, p < 0.05 compared to radiation only (one-factor ANOVA). **C)** Experiment 2 – the presence and size of tumors were assessed two weeks post-inoculation, and the mice were randomized into four treatment groups. Treatment was
performed during week 4 as described for panel A. D) Representative images from (C) of the urogenital system in non-treated (normal), PC-3M inoculated (Ctrl) and treated (X and X+SR) mice. Samples were collected from mice euthanized 50 to 70 days post-grafting.
Figure 2
Figure 3

A
Surviving Fraction

B
C4-2B

X-rays (Gy)

0
0.1
0.01

1
1

0

LNCaP

X-rays (Gy)

0
0.1
0.01

1
1

0

Figure 3
Figure 5

A

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WB:
- DNA-PK
- pS2056
- PARP
- actin

B

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Relative Caspase 3/7 Activity

Figure 5