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Award Number: W81XWH-08-1-0114

TITLE: Mechanisms of Radiosensitization by the Neurotensin
Receptor Antagonist SR48692 in Prostate Cancer Models

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REPORT DATE: April 2010

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188		
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1. REPORT DATE (DD-MM-YYYY) 01-04-2010		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 APR 2009 - 31 MAR 2010	
4. TITLE AND SUBTITLE Mechanisms of Radiosensitization by the Neurotensin Receptor Antagonist SR48692 in Prostate Cancer Models			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER W81XWH-08-1-0114		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Jaroslaw Dziegielelewski Nicholas C.K. Valerie, John DaSilva, Eli V. Casarez, Amol S. Hosing, Marya E. Dunlap-Brown, Sarah J. Parsons, James M. Larner			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Virginia Charlottesville VA 22904			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release					
13. SUPPLEMENTARY NOTES					
ABSTRACT: This project tests the hypothesis that blocking NTR1 receptor with antagonist SR48692 could selectively sensitize prostate cancer cells to ionizing radiation, thus improving outcomes of radiotherapy. In this second year period, we studied the molecular mechanism of radiosensitization using several prostate cancer cell lines differing in the expression levels of NTR1 receptor, EGFR receptor and androgen receptor (AR). We demonstrated that SR48692 selectively sensitizes prostate cancer cells but not normal epithelial cells, due to differences in NTR1 expression. We also demonstrated that the level of radiosensitization depends on EGFR expression in cells. However, sensitization does not depend on AR expression, suggesting that SR48692 treatment could be used to enhance radiotherapy of both androgen-dependent and independent tumors.					
15. SUBJECT TERMS radiotherapy, prostate cancer, drug development, neuroendocrine signaling, neurotensin					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 8	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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April 2010

**Project: Mechanisms of Radiosensitization by the Neurotensin Receptor Antagonist SR48692
in Prostate Cancer Models**

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INTRODUCTION

Cancer of the prostate is the most common cancer in men in the United States and the second leading cause of cancer deaths. Radiation therapy, often in combination with androgen ablation, is commonly administered for patients in the early (androgen-dependent) stage of the disease. Although the vast majority of these primary prostate tumors initially respond to treatment, local failures are not uncommon and frequently lead to androgen-independent and highly metastatic disease. The appearance of neuroendocrine (NE) cells in prostate tumors is associated with progression of the disease, due to their ability to secrete neuropeptides, including neurotensin (NT), that act in a paracrine fashion to promote the growth, survival, and migration of surrounding prostate cancer (PC) cells. PC cells can also secrete these neuropeptides, which function in an autocrine loop. Since PC cells express neurotensin receptor type 1 (NTR1) they respond to NT stimulation by increased growth, DNA replication, migration and enhanced survival. We have hypothesized that neurotensin (and other neuropeptides) contribute to tumor progression and resistance to therapy and blocking it will result in radiosensitization of the cancer cells.

In the previous, first year of this project we have demonstrated that SR48692, a selective antagonist of NTR1 radiosensitizes both PC cells in vitro and PC xenografts in vivo. Importantly, we have shown that SR48692 does not radiosensitize normal prostate epithelial cells.

BODY

Since our previous work (see 1st year annual report) clearly established that blocking NTR1 with SR48692 sensitizes prostate cancer cells to ionizing radiation, in vitro (Task 1a and 1b, months 1-12) and in animal models (Task 2a, 2b, and 2c, month 1-24), during this phase of the project we have concentrated on studying the molecular mechanism of SR48692-directed radiosensitization (Task 4, month 1-36). The signaling pathways mediating the effects of NT on prostate cells are numerous, but a critical downstream pathway is the EGFR/Src/STAT signaling axis (Amorino et al., 2007), which could, in turn, activate downstream transcription factors, including AR.

Role of NTR1 receptor in SR48692-induced radiosensitization: We have compared the radiosensitizing properties of SR48692 in a prostate cancer cell line (PC-3M, highly metastatic human prostate adenocarcinoma cells) and apparently normal human prostate epithelial cells (RWPE-1). Following 24 h treatment with 1 μ M drug, the cells were irradiated with x-ray doses with a range of 0 to 6 Gy and re-plated for colony formation. Colonies containing more than 50 cells were fixed, stained and scored, and the surviving fraction was calculated according to the standard method.

As shown in **Fig. 1A**, SR48692 effectively sensitizes PC-3M prostate cancer cells to ionizing radiation at all studied doses. Importantly, pre-treatment with SR48692 does not have any significant effect on radiosensitivity of normal epithelial cell line RWPE-1, as shown in **Fig. 1B**.

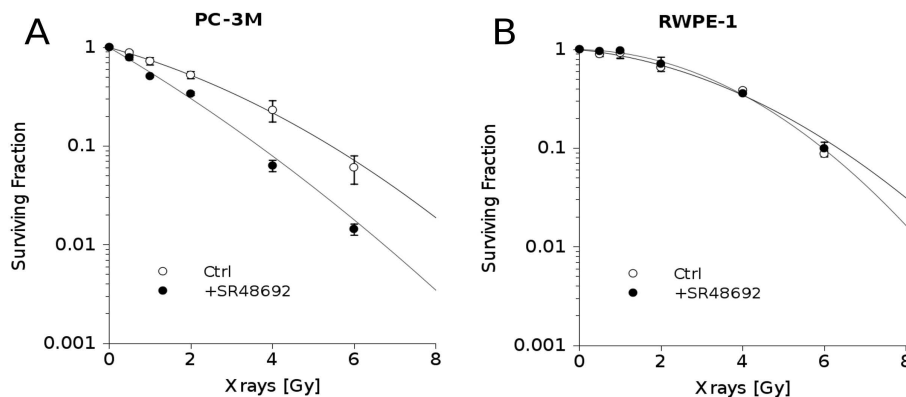


Figure 1. SR48692 sensitizes human prostate cancer cells (A, PC-3M) to ionizing radiation in vitro, but does not have any significant radiosensitizing effect in normal prostate epithelial cells (B, RWPE-1).

The difference between these two cell lines can be explained by our hypothesis that only cells expressing NTR1 will respond to SR48692 treatment. **Fig. 2** clearly demonstrates that normal prostate epithelial RWPE-1 cells do not express NTR1, while prostate cancer cells (PC-3M, PC-3, DU-145, C4-2 and LNCaP) express it. Since there is evidence that the majority of human prostate tumors express NTR1, while normal surrounding tissues do not, SR48692 could become a clinically important radiosensitizer for prostate radiotherapy.

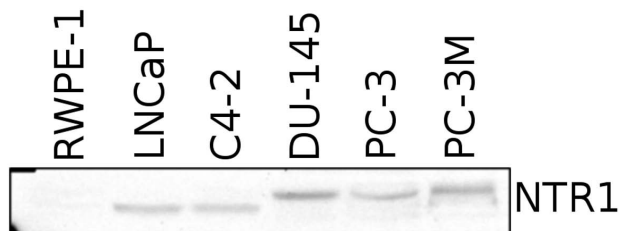


Figure 2. Expression of NTR1 in normal (RWPE-1) and PC cell lines (LNCaP, C4-2, DU-145, PC-3 and PC-3M).

Amorino et al. (2007) demonstrated that NT treatment induces Src-dependent EGFR phosphorylation/activation in PC-3 cells and that this activation can lead to enhanced growth rate and survival of cancer cells. Here (**Fig. 3A**) we confirmed that observation and demonstrated that SR48692 can completely block this stimulation, but has no effect on normal RWPE-1 cells (**Fig. 3A and 3B**).

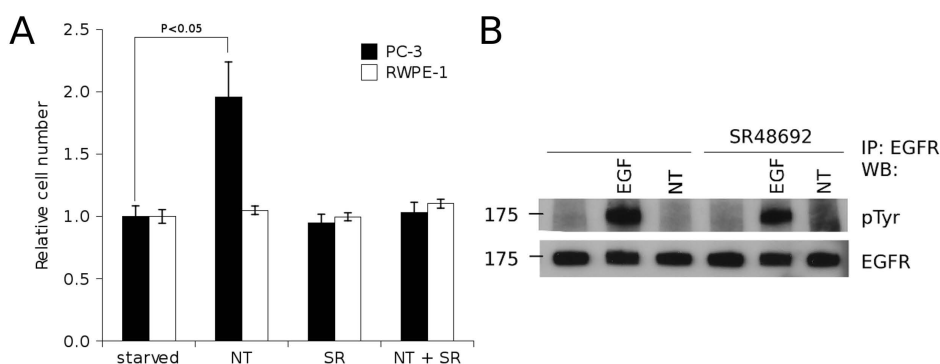


Figure 3. Neurotensin stimulates the growth of PC-3M prostate cancer cells but not RWPE-1 normal prostate endothelial cells. (A) PC-3M and RWPE-1 cell growth following treatment with NT and blocking with SR48692. (B) EGFR phosphorylation in RWPE-1 is induced by EGF but not by NT treatment.

Role of AR receptor in SR48692-induced radiosensitization: We have compared the radiosensitizing activity of SR48692 in PC-3M (AR-negative) and C4-2 (AR-positive) cells. Cells growing in complete medium were treated with SR48692 (1 μ M/24h) and irradiated as described for Fig. 1. Surviving fractions were calculated based on colony forming assay and results presented in **Fig. 4A and 4B**. Although the extent of radiosensitization varies between these two cell lines, there is no qualitative difference in SR48692 activity in regard to AR-expression (**Fig. 4C**).

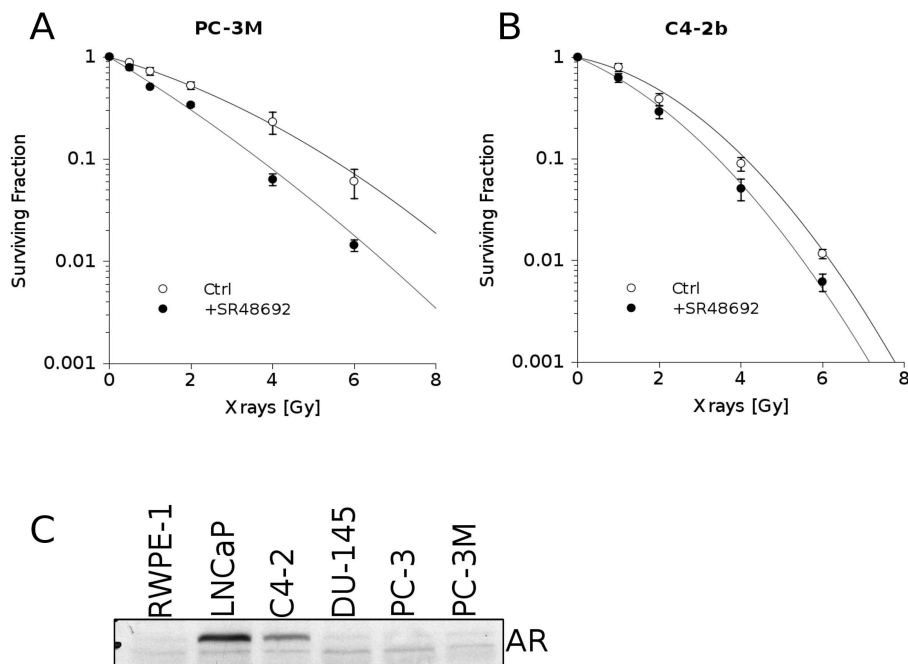


Figure 4. SR48692 radiosensitizes human prostate cancer cells independently of their androgen receptor status (**A**, PC-3M, AR-) and (**B**, C4-2B, AR+). (**C**) Expression of AR in normal (RWPE-1) and PC cell lines (LNCaP, C4-2, DU-145, PC-3 and PC-3M).

However, we have discovered that NT treatment enhances AR stabilization and phosphorylation in LNCaP cells (AR-positive and androgen-dependent), and this effect can be abrogated by treatment with SR48692 (**Fig. 5**). LNCaP cells were serum-starved and subsequently stimulated for the indicated times with NT (100 nM), synthetic androgen R1881 (0.1nM), or a combination of the two, and the lysates were analyzed by immunoblotting and/or immunoprecipitation.

This observation is extremely significant, since it demonstrates that AR can mediate growth factor signaling in absence of androgen, thereby promoting androgen-independent growth.

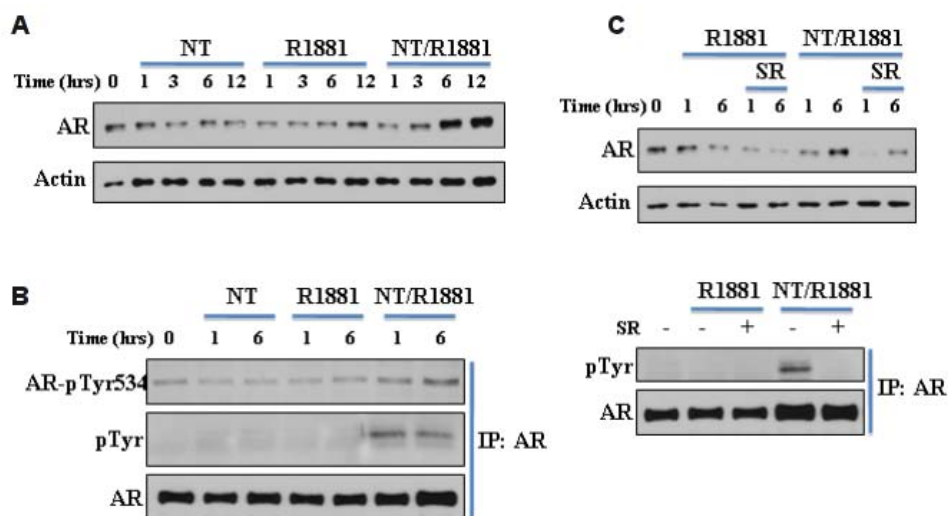


Figure 5. Neurotensin/NTR1 signaling enhances AR (Y534) phosphorylation and stabilization of AR protein. (**A**) NT treatment stabilizes AR protein. (**B**) NT treatment enhances androgen-induced AR phosphorylation (Y5534). (**C**) SR48692 inhibits NT-induced AR phosphorylation and stabilization.

Role of EGFR receptor in SR48692-induced radiosensitization: According to our initial model, EGFR plays a central role in signal transduction downstream of from NTR1. We tested the possibility that not only presence and activity of EGFR, but also the relative level of EGFR protein expression can influence radiosensitizing activity of SR48692. This is a very important question in prostate cancer therapy since a wide variation in EGFR expression is observed in clinical biopsies. PC-3M (low EGFR expression) and DU-145 (high EGFR expression) cells were used in radiation survival clonogenic assays, following 24h pre-treatment with SR48692 (as described above). **Fig. 6** shows that SR48692

radiosensitizes only PC-3M (**Fig. 6A**) cells, which express low levels of EGFR protein, but not DU-145 (**Fig. 6B**), which over-express EGFR (**Fig. 6C**).

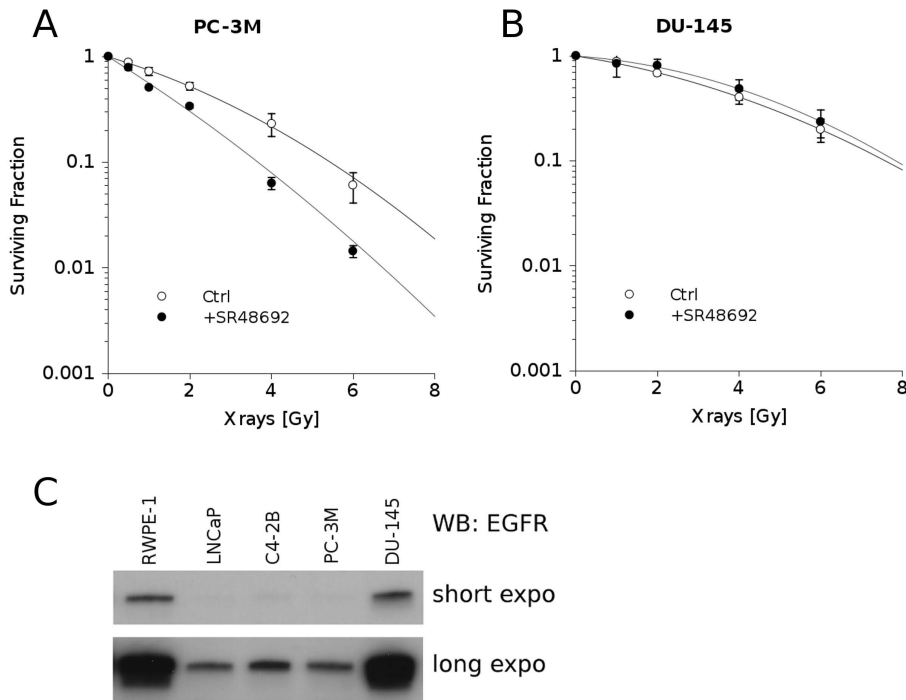


Figure 6. SR48692 radiosensitizes human prostate cancer cells expressing low levels of EGFR (**A**, PC-3M) but not prostate cancer cells overexpressing EGFR (**B**, DU-145). (**C**) Expression of EGFR in normal (RWPE-1) and PC cell lines (LNCaP, C4-2, DU-145, PC-3 and PC-3M).

In summary, our data show that SR48692 selectively sensitizes PC cells to ionizing radiation. Our results also show that NT stimulation (a) activates a novel EGFR/Src/Stat5b signaling pathway and enhances PC cell proliferation and (b) stabilizes the androgen receptor (AR) through EGFR/Src-dependent phosphorylation; both of which can be inhibited by SR48692. Activation of the EGFR, Src and AR pathway(s) has been implicated, not only in the development of androgen-independent disease, but also in tumor metastasis, especially in bones. Future research, planned for the third and final year of this project, will concentrate on animal studies (completing Task 2 and 3), the role(s) of neuroendocrine cell secretions (Task 1c), and will finalize studies on molecular mechanisms of SR48692 radiosensitizing activity (Task 4). In addition, Cetuximab (C-225/Erbitux, ImClone), a clinically used EGFR inhibitor, will be used to study the role of EGFR in SR-induced radiosensitization. We speculate that inhibitors, such as Cetuximab or Dasatinib (Bristol-Myers-Squibb; a Src family inhibitor), will significantly improve experimental radiotherapy outcome and could establish the basis for future combined treatment therapy in humans.

KEY RESEARCH ACCOMPLISHMENTS

- We have demonstrated that radiosensitizing activity of SR48692 depends on the expression of NTR1 receptor in prostate cells. This establishes the foundation of cancer-specificity of SR48692 radiosensitizing activity.
- We have demonstrated that radiosensitizing activity of SR48692 is not dependent on androgen receptor (AR) expression levels in prostate cells. However, SR48692 blocks neurotensin-induced AR phosphorylation/stabilization. Therefore, blocking NTR1 receptor could provide additional benefits to anti-tumor therapy.
- We have demonstrated that the radiosensitizing activity of SR48692 is affected by the EGFR receptor levels in prostate cells.

REPORTABLE OUTCOMES

The results obtained during two years of this project were presented at the 55th Annual Meeting of the Radiation Research Society [1] and 51st Annual Meeting of American Society for Therapeutic Radiology and Oncology [2], and the Gordon Research Conference on Radiation Oncology [3]. In addition a presentation describing the recent discoveries was accepted for this year annual meetings of Radiation Research Society [4].

The new insight into the role(s) of NTR1 in prostate carcinogenesis as well as role(s) of neuroendocrine cells in cancer progression and resistance to radiation gained in this project were the basis for a new grant application: "Effects of prolonged exposure to space radiation on carcinogenesis and neuroendocrine differentiation in human prostate models", submitted to the NASA Human Research Program. Following a very favorable scientific review (score 97 out of 100), the application was funded for 3 years [5]. Additional grant applications based on, and further developing this project, have been recently submitted [6] or are in preparation [7].

[1] Dziegielewski J, Pemberton BL, Dunlap-Brown ME, Lerner JM, Parsons SJ & Amorino GP. SR48692, a specific neurotensin receptor (NTR1) antagonist, sensitizes prostate cancer cells to ionizing radiation, in both in vitro and in vivo models. 55th Annual Meeting Radiation Research Society, Savannah, GA, 2009 (poster and oral presentation).

[2] Dziegielewski J, Pemberton BL, Dunlap-Brown ME, Parsons SJ, Amorino GP & Lerner JM. The specific neurotensin receptor (NTR1) antagonist, SR48692, sensitizes prostate cancer cells to ionizing radiation, in both in vitro and in vivo models. 51st ASTRO Annual Meeting, Chicago, IL, 2009.

[3] Dziegielewski J, Valerie NCK, Casarez EV Jr., Dunlap-Brown ME, Hosing AS, Parsons SJ & Lerner JM. Targeting neurotensin receptor 1 (NTR1) signaling pathway(s) sensitizes prostate cancer cells to ionizing radiation. Gordon Research Conference – Radiation Oncology, Galveston, TX, 2010

[4] Dziegielewski J, Valerie NCK, Dasilva JO, Hosing AS, Parsons SJ & Lerner JM. Interplay between neurotensin receptor 1 (NTR1) and epidermal growth factor receptor (EGFR) in responses to ionizing radiation in prostate cells. 56th Annual Meeting Radiation Research Society, Maui, HI, 2010.

[5] Dziegielewski J (Principal Investigator). Effects of prolonged exposure to space radiation on carcinogenesis and neuroendocrine differentiation in human prostate models. NASA Ground-Based Studies in Space Radiobiology Award NNX10AC13G (2010-2013).

[6] Lerner JM (PI), Parsons SJ (co-Investigator) & Dziegielewski J (co-Investigator). Blocking neurotensin signaling as a novel treatment strategy for prostate cancer – translation from basic science to clinical use. NIH American Recovery & Reinvestment Act grant proposal, submitted March 2010.

[7] Dziegielewski J (PI). Molecular mechanism of NTR1 antagonist – induced radiosensitization in prostate cancers. To be submitted to NIH in June 2010.

CONCLUSION

The results obtained during the second year of this project demonstrate that SR48692, a specific inhibitor of NTR1 receptor, sensitizes human prostate cancer cells to ionizing radiation in AR-independent and EGFR-dependent manner. It does not, however, sensitize normal prostate epithelial cells. Based on these observations, we strongly believe that SR48692 treatment could improve the outcomes of radiotherapy in patients with both early (AR-dependent) and late (AR-independent) stages of prostate cancers. Since EGFR expression levels seem to play an important role in SR48692 activity, future studies will include combinations of radiotherapy with NTR1 and EGFR inhibitor treatment.