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PRINCIPAL INVESTIGATOR: Christopher Willey, M.D., Ph.D.

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37232-5671

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6. **AUTHOR(S)**  
Christopher Willey, M.D., Ph.D.  
Email: christopher.willey@vanderbilt.edu  

7. **PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**  
Vanderbilt University Medical Center  
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14. **ABSTRACT**  
Radiation-induced activation of the PI3K/Akt signal transduction pathway requires Akt binding to phosphatidylinositol phosphates (PIPs) on the cell membrane. The tyrosine kinase, bone marrow X-kinase (Bmx), binds to PIPs in a manner similar to Akt. Since Bmx is known to be involved in cell growth and survival pathways, Bmx could contribute to the radiation response within the vascular endothelium and prostate cancer, which highly express this protein. We therefore are studying Bmx signaling within the vascular endothelium and prostate cancer. Our initial studies have focused on the vascular endothelium. Bmx was activated rapidly in response to clinically relevant doses of ionizing radiation. Bmx inhibition enhanced the efficacy of radiotherapy in endothelial cells within in vitro systems. Retroviral shRNA knockdown of Bmx protein enhanced HUVEC radiosensitization. Furthermore, pretreatment of HUVEC with a pharmacological inhibitor of Bmx, LFM-A13, produced significant radiosensitization of endothelial cells as measured by clonogenic survival analysis and apoptosis as well as functional assays including cell migration and tubule formation. In vivo, LFM-A13, when combined with radiation resulted in significant inhibition of blood flow within prostate tumors as measured by pixel weighted power doppler. Bmx, therefore, represents a molecular target for the development of novel radiosensitizing agents.  

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INTRODUCTION

Patients with intermediate to high risk prostate cancer have a 30% risk of local recurrence despite receiving radiation. Hormone ablative treatment can improve the outcome including survival, especially when combined with regional radiation coverage. This suggests that the biological modification by hormone deprivation sensitizes the prostate cancer cells to radiation. In addition, dose escalation has been shown in a phase III trial to result in improved tumor control which further emphasizes the potential for improving outcomes by optimizing local control of prostate cancer. Therefore, this new strategy to sensitize prostate cancer to radiation treatment is likely to translate into a significant survival benefit for patients afflicted with this disease. The tumor microvasculature is also a target for prostate cancer treatment. Both clinical and basic science research have identified the tumor microvasculature as a major factor that supports tumor growth. Moreover, this microvasculature is thought to contribute to the radiation resistance seen in many solid tumors. In order to improve outcomes, efforts aimed at improving local control through targeting of tumor vasculature have been undertaken. Our laboratory has studied the inherent resistance of the tumor vascular endothelium to the cytotoxic effects of IR. We have found that IR induces the activation of Bmx which regulates endothelial cell viability and function. Our preliminary studies show that radiation induces Bmx activation in a similar time course to PI3K/Akt activation and that a small molecule inhibitor of Bmx modulates the cellular viability of endothelial and prostate cancer cells, particularly with radiation. The goals of the proposed research study are to determine whether Bmx inhibition can enhance the cytotoxic effects of radiation in these endothelial and prostate cancer cells as well as to identify the mechanism by which Bmx is activated by radiation. Because this is the first description of Bmx activation by radiation, this research will potentially lead to a new molecular target for radiation sensitization. Our ultimate goal is to bring this therapeutic strategy into clinical trials.

BODY

Statement of Work - Examining Bmx within prostate cancer and tumor vasculature

Task 1. To determine the activation profile of Bmx in response to radiation in endothelial and prostate cancer cells and determine if Bmx inhibition can enhance radiation efficacy in these cells (Months 1-14):

The goal of this task is to characterize Bmx within in vitro model systems of both prostate cancer and vascular endothelium. The bulk of the work thus far has been performed within human umbilical vein endothelial cells (HUVEC) which are a model of the vasculature. One of the advantages of HUVEC is that they are pooled primary cultured cells from several human donors. As such, significant findings in these cells are more likely to have broad application for patients due to the heterogeneity. We have already accomplished most of the objectives that were proposed for this task, though we still need to test the prostate cancer cell lines using the same techniques.

a) Immunoprecipitate Bmx from irradiated endothelial cells and prostate cancer cells lines and assay them for kinase activity (Months 1-6).

Bmx is activated in endothelium upon irradiation

We examined primary culture vascular endothelial cells (HUVEC) to determine whether Bmx was activated by ionizing radiation, because of its similarities in structure and signaling with that of Akt, and was possibly contributing to radiation resistance. Figure 1A demonstrates a time course of Bmx activation upon irradiation with a clinically relevant dose of 2 Gy. Tyrosine 40, present in the PH domain of Bmx, becomes phosphorylated during its activation. Bmx is phosphorylated at 60 min. following 2 Gy of irradiation. To confirm this finding, we utilized an in vitro kinase (IVK) assay in which Bmx was immunoprecipitated from irradiated or sham irradiated endothelial cells and then incubated with ATP in a kinase reaction. These samples were then run on SDS-PAGE and probed for anti-phosphotyrosine to analyze autophosphorylation of Bmx. As shown in figure 1B, Bmx was activated after irradiation. Examination of total Bmx revealed no change in Bmx levels at any of the time points that were assayed. Densitometric quantitation (mean and standard errors) from four separate experiments is shown as well. Interestingly, Bmx showed significant kinase activity immediately following irradiation and then has a second peak of lesser activity at 1h.
**b) Construct shRNA retrovirus vectors specific for Bmx, express them in endothelial cells, and assay endothelial cell function with radiation (Months 1-12).**

**Bmx knockdown enhances radiation efficacy in endothelium**

Because we were able to detect a clear activation of Bmx following a clinically relevant dose of IR, we wanted to determine whether Bmx activation protects the endothelial cells from cytotoxic damage. Since primary culture endothelial cells, such as HUVEC, have low transfection yields, we utilized a retroviral shRNA system to knockdown Bmx levels prior to irradiation. Figure 2A shows five different retroviral constructs (A through E) for Bmx as well as a negative control construct (Neg) that were used to infect HUVEC. After 48 h, infected cells were harvested and lysates were prepared for total Bmx Western blotting. As can be seen, construct A (shBmxA) provided ~90% knockdown of Bmx protein levels compared to the negative control shRNA vector. Bmx knockdown experiments were performed with or without irradiation. Figure 2B shows MTT-based (WST-1) survival assay. HUVEC infected with either shBmxA or negative control vectors. After 48 h, cells were counted and plated at 10,000 cells/well in duplicate within 96-well dishes. The cells were treated with either sham (0 Gy) or 2 Gy irradiation and incubated for 24h. Following this incubation, WST-1 labeling mixture was added to each well and analyzed at OD450 nm to determine mitochondrial viability. Normalized values for OD450 nm are shown as mean and standard error. Combined Bmx knockdown with irradiation decreases HUVEC survival.
c) Construct stable prostate cancer cell lines that express inducible shRNA to Bmx and assay these cell lines for enhancement of radiation effect (Months 1-14).

We had significant difficulty in producing inducible shRNA vectors for Bmx. As detailed in part b) above, we have found an acceptable shRNA vector from a commercial source (Origene) for Bmx knockdown in HUVEC, but we have tried to generate similar constructs within an inducible retroviral vector, but have not been successful as of yet. We are still trying to construct an inducible system, but may be required to use the non-inducible shRNA vectors that we successfully used in HUVEC. The non-inducible shRNA vectors shown in figure 2 do have mammalian antibiotic resistance such that we can select for stable clones in the prostate cancer cell lines DU145, LNCaP and PC3 using puromycin selectivity.

d) Characterize the efficacy of Bmx specific inhibitors in enhancing radiation effect on endothelial cells and prostate cancer cells (Months 1-14).

As with the other sub-tasks for Task 1, our initial focus has been to characterize HUVEC in terms of Bmx specific inhibitors. Due to the more extensive published data utilizing LFM-A13 in both in vitro and in vivo systems, we have chosen to study this drug in greater detail. The other inhibitors that have been considered include terreic acid and AG879. However, we have not completed the full set of proposed experiments for those drugs. The terreic acid appears to be the more toxic of the three drugs.

Other consideration related to this task which was proposed as part of the future goals in the original proposal includes the identification of novel Bmx inhibitors through Vanderbilt University Institute of Chemical Biology. Most importantly, we are pursuing a collaboration with the Department of Pharmacology to try to develop these Bmx inhibitors in hopes of producing better and novel agents that can eventually be tested in vivo.
However, for this annual report, the data presented below detail the use of the commercially available drug, LFM-A13 within HUVEC. We still need to perform similar studies within prostate cancer cells.

**Pharmacological inhibition of Bmx**
Having established that Bmx knockdown can enhance radiation efficacy in endothelial cells, we wanted to determine whether or not pharmacological inhibition of Bmx would show the same effect. Bmx specific inhibitors \(^6\)\(^-\)\(^9\) have been described, particularly LFM-A13, which targets the Tec family. Since Bmx is the only Tec family member expressed in endothelium, we studied this drug in HUVEC. The drug, LFM-A13 has been shown to block VEGF induced signaling through Bmx inhibition in HUVEC at a dose of 25 µM. Therefore, we utilized 30 µM LFM-A13 for *in vitro* studies. Figure 3A shows 3 µM (subtherapeutic) vs. 30 µM LFM-A13 pre-incubation on radiation-induced Bmx activation in the *in vitro* kinase assay at the time points with highest Bmx activation. As can be seen, 30 µM but not 3 µM LFM-A13 attenuates the activation of Bmx in response to 3 Gy.

**Bmx inhibition attenuates endothelial cell viability**
To determine whether LFM-A13 produces a radiosensitization effect in HUVEC, we studied clonogenic survival assays in HUVEC with LFM-A13 pre-incubation (Figure 3B). HUVEC were pre-treated with DMSO vehicle control or 30 µM LFM-A13 45 min prior to irradiation with 0, 2, 4, or 6 Gy. Colonies were allowed to form over 10 days which were then counted and the surviving fraction was calculated for each radiation dose. These studies indicated that 30 µM LFM-A13 can radiosensitize HUVEC compared to the control as evidenced by the statistically significant downward survival curve shift. The dose enhancing ratio (DER) was 1.47.

Apoptosis was studied to determine whether this is a mechanism of enhanced cytotoxicity. Figure 3C illustrates the effect of LFM-A13 on apoptosis within these cells. HUVEC treated with 30 µM LFM-A13 or DMSO control were subjected to sham or 3 Gy irradiation and then incubated for 24 h prior to trypsinization and flow cytometric analysis. Annexin V-propidium iodide staining revealed that drug or 3 Gy alone was not capable of shifting cells into either early (Q4-1) or late (Q2-1) apoptosis but that the combination of LFM-A13 and 3 Gy caused a statistically significant (*p*<0.001 vs. LFM-A13 or 3 Gy alone) increase in apoptosis. To confirm these findings, HUVEC were treated with either 30 µM LFM-A13 or DMSO control with or without 3 or 6 Gy irradiation and incubated for 24 h. These cells were fixed and stained with DAPI and the percent of apoptotic cells was quantified. As shown in Figure 3D, the combination of LFM-A13 and irradiation resulted in enhancement of apoptosis (* indicates *p*<0.05 vs. DMSO control and ** indicates *p*<0.001 vs. LFM alone).
Figure 3. Radiation-induced endothelial cell cytotoxicity is enhanced by Bmx inhibition. A) In vitro kinase assay (IVK) for LFM-A13. Bmx was immunoprecipitated (IP:Bmx) from lysates of HUVEC pre-treated with either 3 or 30 μM LFM-A13 for 60 min prior to 3 Gy irradiation and harvested at the indicated times. IP:Bmx samples were then eluted in non-denaturing conditions and subjected to kinase assay. Samples were then loaded for SDS-PAGE and anti-phosphotyrosine (IB:PY) Western blotting. Total Bmx levels were determined by anti-Bmx Western blotting (IB:Bmx). B) Clonogenic assay of HUVEC cells with 1 hour preincubation with 30 μM LFM-A13 or DMSO vehicle control is shown. Cells were counted and plated and subjected to indicated doses of radiation and colonies formed over 10 days. Surviving colonies were plotted as a function of cells plated and normalized by the plating efficiency for each condition (Shown as surviving fraction, SF). SE Bars are shown. (*, p=0.007; **, p=0.0002) C) 30 μM LFM-A13 vs. DMSO vehicle control was added to plated cells with or without 3 Gy radiation (IR) 60 min later. After 24h, cells were trypsinized and collected for flow cytometry using Annexin V/Propidium iodide staining. Percent cells undergoing early (Q4-1) and late (Q2-1) apoptosis were compared to viable cells (Q3-1) and dead cells (Q1-1) as indicated. Quantification of early + late apoptosis is shown graphically as mean and standard error. (*, p<0.001 vs. LFM-A13 or 3 Gy alone) D) Cells preincubated 60 min with 30 μM LFM-A13 or DMSO vehicle control were treated with either 3 or 6 Gy of irradiation and incubated at 37° C for 24 h prior to fixing and staining with DAPI. Percent of cells demonstrating apoptotic morphology (marked by arrows) was calculated and is shown as mean and standard error. (*, p<0.05 vs. control; **, p=0.001 vs LFM-A13 alone).
Bmx inhibition attenuates endothelial cell function

Functional assays of endothelial cells include cell migration and capillary-like tubule formation. Figure 4A illustrates the effect of LFM-A13 and irradiation on endothelial migration across a gap (endothelial cell closure assay) at 12 and 24 h. HUVEC were plated on glass slides and grown to 80% confluency. A gap region, free of cells, was then created using a 200 μl pipette tip. These slides were then treated with 30 μM LFM-A13 or DMSO control for 45 min prior to either 0 or 3 Gy. Cells were fixed and stained at 12 or 24 h and photographs were taken of the gap region and the surrounding cells to determine the ability of the HUVEC to migrate across and fill the gap. Relative cell density was calculated for each condition to control for the cytotoxic effects of treatment as shown in Figure 4B. By 24 h, control cells effectively migrated across the gap. 30 μM LFM-A13 or 3 Gy alone had minimal effect on attenuating endothelial cell closure at both 12 h and 24 h compared to vehicle treated control. However, the combination induced a greater than additive effect which was statistically significant (* indicates p<0.05 vs. control, and ** is p<0.01 vs. LFM-A13). Figure 4C and D show capillary tubule formation assay. HUVEC plated onto matrigel were treated with 30 μM LFM-A13 or DMSO with or without 3 Gy irradiation and allowed to form tubules. The cells were then fixed and stained. The number of tubules were quantified and plotted. Representative photographs are shown in Figure 4C and quantified in Figure 4D. Cells that were treated with both LFM-A13 and 3 Gy showed a significant reduction (p<0.005) in tubules formed compared to cells treated with either treatment alone.

Figure 4. Endothelial cell function is attenuated by Bmx inhibition and radiation. A) Endothelial cell closure assay is shown in which 80% confluent HUVEC were subjected to a gap formation using a 200 μl pipette tip. Cells were then treated with 30 μM LFM-A13 or DMSO vehicle control for one hour followed by 3 Gy. 12 and 24 h later, cells were fixed with 70% ethanol and stained with methylene blue. Shown are representative photographs. B) A bar graph of the mean and standard error of relative cell density in the original gap area (n=4) is shown. (*, p<0.05 vs. control; **, p<0.01 vs. LFM-A13 alone) C) HUVEC were placed onto Matrigel plugs and were treated with either 30 μM LFM-A13 or DMSO vehicle control for 30 min followed by 3 Gy irradiation. The cells were then incubated and tubules were allowed to form as shown by the representative photographs. The cells were then fixed and tubules were quantitated by NIH ImageJ software. The mean number of tubules are shown in D) with standard error bars. (*, p<0.05 vs. control; **, p<0.005 vs. LFM-A13 or 3 Gy alone)
Task 2. To determine the mechanism of radiation induced activation of Bmx (Months 3-18).

This task has had minimal progress, thus far, as we have spent most of our efforts on Task 1. In terms of reagents to accomplish the sub-tasks for Task 2, we do have both adenoviruses for c-Src and PI3K and specific drugs that can be utilized. However, for Task 2b, we still need to construct the domain mutants for Bmx. Once the mutants are constructed, they can be transfected into the prostate cancer cell lines, DU145, LNCaP and PC3, but in order to have efficient expression in HUVEC, we will need to place those mutants within an adenovirus or lentiviral vector system. We have viral infection to be required for HUVEC as we have tried several different transfection methods, including electroporation (Amaza system) and New England Biolabs HUVEC TransPass reagent, which have only resulted in significant toxicity to the cells and very little transfection efficiency. Therefore, this task will take significantly longer to accomplish due to the time and expense of creating viruses. As such, the descriptions below detail the proposed studies for these sub-tasks.

a) Treat endothelial cells and prostate cancer cell lines with specific inhibitors (drugs and adenoviruses) to c-Src and PI3K and analyze for attenuation of radiation-induced Bmx activation (Months 3-18).

Several experiments will be undertaken. First, we will treat HUVEC with pharmacological inhibitors of c-Src (SU6656) and PI3K (IC486068) prior to irradiation and then probe for Bmx activation using the Bmx immunoprecipitation with in vitro kinase assay as shown in Figure 1. We and others have previously published data using specific inhibitors for these kinases.10-15 A second set of experiments planned for this sub-task involves the use of dominant negative c-Src, dominant negative PI3K, and control GFP adenoviruses that we already possess in our lab. We will express these adenoviruses in HUVEC and probe for Bmx activation in response to radiation. The last set of experiments will focus on the various prostate cancer cell lines (DU145, LNCaP and PC3).

b) Construct a series of Bmx functional domain mutants, express them in endothelial cells and prostate cancer cell lines, and analyze for attenuation of radiation-induced Bmx activation (Months 3-18).

We will transfect into prostate cancer cell lines and infect into HUVEC, Bmx and Bmx mutants with immunoaffinity tags (hemaglutinin) that we generate from our expression plasmid vector system to determine which functional domain(s) are critical for radiation induced activation. Several groups have used this technique to characterize Bmx interaction with other proteins.16-20 The following point mutations will be generated using site directed mutagenesis: 1) Kinase dead – K444Q16, 18; 2) Dominant negative – K444Q, E42K16; 3) Constitutive membrane association – E42K19; 4) PH domain mutant – R29C19; 5) SH2 domain mutant – R322V17; 6) SH3 domain mutants – Y215F21. We plan to use immunoprecipitation of the immunoaffinity tag with subsequent in vitro kinase assay similar to what is shown in Figure 1 in order to determine activity.

Task 3. To determine whether specific inhibitors of Bmx enhance the therapeutic efficacy of radiotherapy in prostate cancer tumor models (Months 9-24).

For this task, we have begun initial experiments looking at therapeutic potential within an in vivo model system. Because we have now demonstrated that Bmx inhibition sensitizes the vascular endothelial cells to ionizing radiation as shown in the Task 1 report, we have initiated studies looking at the effect of Bmx inhibition within prostate cancer xenografts in athymic nude mice. The other proposed studies for this task are in development at this time and are briefly mentioned below as well.

a) Perform in vivo testing of tumor response to Bmx inhibitors and radiation in human prostate cancer xenografts in athymic nude mice (Months 9-24).

Bmx inhibition attenuates tumor blood flow
To test the effects of Bmx inhibition in vivo, LFM-A13 was used to treat mice harboring DU145 prostate tumor xenografts. Power-weighted pixel density measurements using Doppler sonography showed that ionizing
radiation combined with LFMA13 resulted in a dramatic decrease in tumor blood flow compared to DMSO vehicle control or either treatment alone as shown in Figure 5.

Additional proposed studies for this sub-task

We plan to implant several prostate cancer cell lines (LNCaP, DU145, PC3) into the hind limbs of athymic nude mice and test the Bmx/Etk inhibitors ability to control tumor growth. We will begin with LFM-A13 which has been the most successful drug thus far. As in Figure 5, the animals will be randomized to four treatment groups: 1) Control group – receives vehicle control daily for five days; 2) Drug alone – receives drug alone for five days; 3) radiation alone – daily 3 Gy radiation for five days; 4) Drug plus radiation – receives drug 30 min to 1 h prior to daily 3 Gy radiation treatment for five days. Tumor volumes will be measured every two days using a skin caliper to measure (a) length, (b) width, (c) depth. Tumor volume will be calculated from the formula \((a \times b \times c)/2\) derived from the ellipsoid formula. Tumor growth delay can be measured with this model to show efficacy.

**b) Perform in vivo testing of tumor response to Bmx inhibitors and radiation in transgenic mice that spontaneously form prostate cancers within the prostate (Months 9-24).**

The prostate epithelial specific long probasin promoter and the SV40 large T antigen (LPB12T-7) transgenic mice express an androgen-dependent promoter (LPB) that causes animals to spontaneously form large vascular tumors within the prostate. These mice form extensive high grade prostate intraepithelial neoplastic (HGPIN) lesions as well as invasive lesions with neuroendocrine differentiation. Because these tumors retain androgen sensitivity, we can also determine the effect of androgen deprivation on Bmx activity and radiation sensitivity. This has clinical significance as patients with intermediate and high risk factors receive androgen ablation during radiation therapy. 

**Proposed Studies**

We will treat these animals with radiation and Bmx inhibitors and analyze for tumor response at the end of a specified period of time. We will analyze the differential effect of treatment on the vascular component of the tumor as well as within the tumor itself. We will perform immunohistochemical staining of the tumor endothelium and analyze frozen tissue samples with immunoprecipitation and Western blot analysis for Bmx as described above. We will perform this in both castrated and noncastrated mice to determine if there is an interaction between androgen receptor signaling and the radiation and Bmx inhibition.
KEY RESEARCH ACCOMPLISHMENTS

- Bmx is activated by clinically relevant doses of ionizing radiation in vascular endothelial cells
- Bmx inhibition with shRNA retroviral knockdown enhances radiation cytotoxicity within vascular endothelial cells
- Bmx inhibition with LFM-A13 enhances radiation cytotoxicity and attenuates vascular endothelial cell function
- Ionizing radiation combined with Bmx inhibition using LFM-A13 attenuates tumor blood flow in mouse model of prostate cancer

REPORTABLE OUTCOMES


NCI Joint Lung and Head/Neck SPORE Travel Award – Charleston, SC, February 2007

Tu T, Thotala D, Hallahan DE, Willey CD. Bmx is a vascular endothelial molecular target for radiosensitization. Poster Discussion. ASTRO Convention, Los Angeles, CA, October 2007.


Lastly, this work has helped me to attain a faculty position as a physician scientist in radiation oncology. I have accepted a position as assistant professor at the University of Alabama at Birmingham in the Department of Radiation Oncology with anticipated start date of July 1, 2008.

CONCLUSION

We have determined that the non-receptor tyrosine kinase, Bmx, is activated by ionizing radiation within vascular endothelium. In addition, the preliminary studies from the training grant proposal as well as review of the literature identify Bmx as highly expressed within prostate cancer and prostate cancer cell lines. Therefore, understanding the role of Bmx within prostate cancer and its vasculature may provide a new strategy for prostate cancer treatment.

Our studies, thus far, have shown that Bmx inhibition, with both pharmacologic agents and molecular manipulation, can enhance the therapeutic effect of ionizing radiation on vascular endothelial cells. Furthermore, examination of prostate cancer xenografts within mice confirm these findings as combined Bmx inhibition with radiation results in significant drop in tumor blood flow. Ongoing studies are focused on better understanding the mechanism behind radiation-induced Bmx activation as well as determining whether Bmx inhibition with radiation can promote better tumor control in mouse models.

In summary, Bmx is a new molecular target for radiation sensitization based on in vitro and in vivo experimentation in vascular endothelium. Ongoing studies using various prostate cancer cell lines will help us to determine whether certain prostate cancers are more susceptible to Bmx inhibition when treated with radiation. Ultimately, clinical evaluation of Bmx inhibitors with radiation will be critical during the development of Bmx as a biological target for therapy.
REFERENCES


APPENDICES
None included