

Hypoxia-selective Compounds for Boron Neutron Capture Therapy

by

Jugal Shah

Submitted to the Department of Nuclear Science and Engineering in partial fulfillment of the requirements for the degree of

BACHELOR OF SCIENCE IN NUCLEAR SCIENCE AND ENGINEERING

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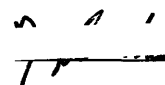
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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Department of Nuclear Science and Engineering
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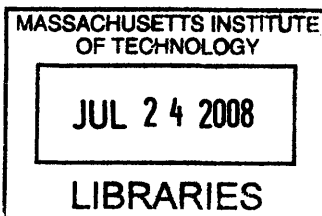
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Abstract

Boron neutron capture therapy (BNCT) is a biochemically targeted form of radiotherapy for cancer. In BNCT, a compound labeled with the stable isotope boron-10 is systemically administered, and tumor cells selectively uptake the boron-10 containing compound at higher concentrations than normal cells. A general problem with the tumor seeking compounds is that drug delivery is dependent upon sufficient vascularization within the tumor. To investigate the possibility of delivering boron to hypoxic regions of tumor, a new boronated nitroimidazole delivery agent has been synthesized as a carrier of boron-10 for BNCT. It is expected that this will be used in combination with the existing boron carrier boronophenylalanine-fructose to treat solid tumors.

An immunohistochemical protocol to visualize hypoxia was tested and refined to confirm the suitability of two tumor models established in the lab for hypoxia related uptake studies. The immunohistochemical protocol is used to detect pimonidazole, which localizes at hypoxic regions in tissue and is the parent compound for the new hypoxia-selective boron carrier. The protocol was used to test and confirm the suitability of a hypoxic *in vivo* tumor model. Two tumor lines were tested: SCCVII squamous cell carcinoma and EMT-6 murine mammary carcinoma. Both exhibited hypoxia. Finally, quantitative studies using Inductive Coupled Plasma Atomic Emission Spectrum demonstrated that the synthesized boronated nitroimidazole reaches suitable concentrations in SCCVII and F98 tumor. Future therapeutic studies are required to empirically confirm the effectiveness of this compound.

Thesis Supervisor: Jeffrey A. Coderre

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**SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS FOR
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CHAPTER ONE

INTRODUCTION

1.1 Introduction to BNCT

1.1.1 Boron Neutron Capture Therapy (BNCT)

Boron Neutron Capture Therapy (BNCT) is a biochemically targeted form of radiotherapy for cancer. In BNCT, a compound labeled with the stable isotope boron-10 is systemically administered, and tumor cells selectively uptake boron-10 at higher concentrations than normal cells (1,2). The disease site is then irradiated with epithermal (0.5 eV-10 keV) neutrons that are captured by the boron-10. The newly created and short-lived boron-11 nucleus fissions by α -decay because it is an unstable isotope, emitting an alpha particle while converting to lithium-7. The alpha particle has 1.47 MeV of energy and the lithium particle has 0.84 MeV of energy. These charged particles, which have a high Linear Energy Transfer (LET), travel only approximately 12-14 μm in tissue, which is about the diameter of a cell (3). Thus energy deposition (i.e. absorbed dose) is restricted primarily to those cells that contained boron. Although the potential exists to destroy tumor cells dispersed in normal tissue parenchyma, effectively providing radiosurgery at the cellular level, clinical progress in the United States is stalled due to the absence of suitable compounds tailored for specific cancers of clinical interest.

1.1.2 Problems with BNCT

A general problem with the tumor seeking compounds is that drug delivery is dependent upon sufficient vascularization within the tumor (4). However, the morphology of most tumors is associated with a dense necrotic and hypoxic core with limited vascularity. Thus, most boron-delivery agents cannot be effectively delivered into a necrotic region. This can impede the effectiveness of therapy because some tumor cells in this hypoxic region drop out of the proliferation phase. These G_0 cells, such named since they no longer proliferate, are radio-resistant and thus capable of re-growth if the target atoms required for BNCT are not delivered to these sites. In principle, BNCT is well suited to treating poorly oxygenated tumors since the reduced radio-sensitivity in the absence of oxygen observed with photons does not apply for high LET radiations. However, to ensure a uniform absorbed dose in all parts of the tumor, there is a need to develop new boron carriers that are less dependent on good blood flow, as is the case for the current compound, boronophenylalanine (BPA), and that are capable of delivering boron to cells in the poorly vascularized and poorly oxygenated regions of the tumor (5).

1.2 Investigating a New Class of Compounds

1.2.1 Introduction to the hypoxia-selective compounds project in BNCT

Tumor hypoxia is generally characterized by an imbalance between oxygen (O_2) supply and consumption that results in low O_2 concentrations, adversely affecting the growth of cells in the region (6). Nitroimidazoles have been widely used as radiosensitizers for photon therapy of hypoxic tumors (7). Nitroimidazoles readily penetrate tumors and produce blood and intratumor concentrations up to 1 mM. They can also exhibit nitroreduction activation under

hypoxic conditions to yield electrophilic species which can form a molecular adduct that is retained in the cell. Coupled with a boron atom, they have the potential to form effective BNCT compounds.

To investigate the possibility of exploiting hypoxic regions of tumor, a new boronated nitroimidazole delivery agent has been synthesized as a carrier of boron-10 for BNCT to be used in combination with the existing boron carrier BPA-F to treat solid tumors. Two different formulations of the compound, hereafter known as compound A and compound B, were synthesized.

1.2.2 Goals of the hypoxia-selective BNCT compound project

The goal of this thesis project is to develop a system that will accurately test whether the use of a nitroimidazole-carborane will provide a more homogeneous distribution of boron in tumor than the current clinically approved boron carrier. It will be tested using squamous cell carcinoma (SCCVII) in mice and F98 rat brain glioma in rats, both of which are known to develop tumors with hypoxic areas. The major tasks of this project are to:

- a) Develop a protocol for hypoxia visualization
- b) Establish tumor hypoxia in mouse models
- c) Quantitatively measure boron concentrations in tumor and non-tumor tissues

The protocol for hypoxia visualization and its development will be described in detail in Chapter 2. The establishment of the mouse model as sufficiently hypoxic will be described in detail in Chapter 3. The use of Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

to measure boron concentrations of new compounds in tissue will be described in Chapter 4.

The hypoxia-selective BNCT compound project will be summarized in Chapter 5.

1.3 References:

1. Brownwell, G., Zamenhof, R., Murray, B., & Wellum, G. (1978). *Boron Neutron Capture Therapy*. New York: Grune and Stratton, Inc.
2. Barth, R., Solloway, A., & Fairchild, R. (1990). Boron Neutron Capture Therapy of Cancer. *Cancer Research* , 1061-1070
3. Archanbeau, A., Alcober, V., Calvo, W., & Brenneis, H. (1964). Biological Effects of Thermal Neutrons and the $[^{10}\text{B}(n, [\alpha])^7\text{Li}]$ Reaction." In: Biological effects of Neutron and Proton irradiations. *Biological effects of Neutron and Proton irradiations*, (pp. 55-70). Vienna.
4. Hatanaka, H. K., Amano, K., Hojo, S., Sano, K., Egawa, S., & Yasukochi, H. (1986). Clinical Experience of Boron Neutron Capture Therapy for Gliomas - A Comparison with Conventional Chemo- Immuno-Radiotherapy. In *Boron Neutron Capture Therapy for Tumors* (pp. 349 -378). Niigata: Nishimura Co., Ltd.
5. Pfeiffer, C., & Jenney, E. (1950). The Pharmacology of Boric Acid and Boron Compounds. *Bull. Natl. Formulary Comm.*, 57-80.
6. Vaupel, P., Harrison, L., (2004). Tumor Hypoxia: Causative Factors, Compensatory Mechanisms, and Cellular Response. *Oncologist* 2004;9;4-9
7. Wood, P., Scobie, M., & Threadgill, M. (1996). Uptake and retention of nitroimidazole-carboranes designed for boron neutron capture therapy in experimental murine tumours: detection by ^{11}B magnetic resonance spectroscopy. *Int. J. Radiat. Biol* , 587-592.

CHAPTER TWO

DEVELOPING A PROTOCOL FOR HYPOXIA VISUALIZATION

2.1 Introduction

Prior to attempting to produce *in vivo* hypoxic models, a protocol to visualize hypoxia was tested and refined for different tissues such as brain, liver, and tumor. The immunohistochemical protocol to detect the presence of hypoxia was adapted from the commercially-available Hypoxyprobe™ kit (pimonidazole hydrochloride).

Other possible measures of hypoxia include the hexafluorinated CCI-103F marker and detecting the transcription factor hypoxia-inducible factor-1 (HIF-1) (1,2). Detection via CCI-103F was not chosen due to the relatively low solubility of the compound at 10 millimolar or less (3). Additionally, CCI-103F requires a relatively complicated intraperitoneal injection dissolved in peanut oil and DMSO. HIF-1 detection was not chosen because of the variability of HIF-1 transcription between hypoxic cells (4).

2.2 Pimonidazole-mediated Hypoxia Detection Protocol

The protocol was adapted from the CHEMICON IHC Select® kit and the Hypoxyprobe™-1 Plus Kit for the Detection of Tissue Hypoxia (5,6). The protocol that will be described is a final

working protocol and has resulted in consistent results. Minor changes in the protocol result in changes in staining intensity.

2.2.1 Overview

The immunohistochemical protocol is used to detect pimonidazole, which localizes at hypoxic regions in tissue. The kit used in the protocol is the CHEMICON IHC Select® Immunoperoxidase Secondary Detection System (Cat. No. DAB150). The protocol is a two-step immunohistochemical procedure using a primary fluorescein (FITC)-conjugated mouse monoclonal antibody (Mab) directed against pimonidazole protein adducts and a secondary mouse anti-FITC Mab conjugated to horseradish peroxidase (HRP). A chromogenic reagent, 3,3' diaminobenzidine, is then added and reacts with the enzymatic action of the HRP, which results in the deposit of brown to black insoluble precipitate, visible under a microscope.

The immunohistochemical protocol assumes the tissue has been formalin-fixed. Thus, tissue antigen-retrieval is a major component of the protocol. Prior to this step, the tissue originates in a mouse or rat, as described in the Hypoxyprobe™ kit. Ninety-minutes prior to the mouse sacrifice, pimonidazole is administered at a dose of 60 mg/kg via an intraperitoneal injection. The tissues of interest, which are usually tumor and liver, are excised from the mouse following euthanasia, cut to a maximum of 3 mm thickness, and immediately submerged in 10.0% neutral buffered formalin to cross-link and preserve tissue. Formalin-fixing occurs for 12-20 hours, after which time the tissue is placed in 70% ethanol for storage. At this point, the tissue is submitted to the MIT Division of Comparative Medicine, where it is embedded in paraffin to harden to allow for thin slicing, sliced to a maximum of 5 µm using a microtome, and mounted to a slide

by heating fixed tissue at 60°C for 60 minutes. At this point, antigen retrieval and immunohistochemical staining can begin.

2.2.2 Tissue Pretreatment

The first step to recover antigens for immunohistochemical staining is to deparaffinize the tissue.

The slides are dipped into Coplin glass jars in the following order for the following durations:

1. Xylene 3 x 3 minutes
2. 100% Ethanol 2 x 1 minute
3. 70% Ethanol 1 x 1 minute
4. 30% Ethanol 1 x 1 minute
5. Deionized Water 1 x 1 minute

Following paraffin removal, the slides are placed in a Coplin jar containing pH 6.0 citrate buffer.

The Coplin jar is placed in a rice cooker filled with water two-thirds up to the top of the Coplin jar and boiled for 10 minutes. The Coplin jar is then removed and cooled on the bench top at room temperature for 10 minutes.

The slides are removed from the Coplin jar. The area around the mounted tissue is blotted dry and circled using a PAP Pen to create a hydrophobic barrier to hold the treatment solutions in place for future steps. Two parallel lines are also drawn with the PAP Pen across the narrow width of each slide, where one line is on each side of the tissue. The PAP Pen barrier is allowed to dry for at least two minutes at room temperature.

To quench any natural peroxidase activity native to the tissue, two or more drops of 3% hydrogen peroxide is added to cover the specimen. Natural peroxidase activity may result in false-positive nonspecific background staining that leads to poor contrast or erroneous results. The slide is allowed to incubate for 10 minutes.

2.2.3 Rinse Procedure

Rinsing buffer is mixed according to the instructions in the CHEMICON kit. Rinse buffer at 1X concentration contains 5% Tris-buffered saline (TBS) and 0.1% optional Tween© 20 buffer.

The following rinse procedure is completed between each incubation step, or as otherwise specified:

1. Pour off solution and tap the edge of each slide onto a paper towel.
2. Holding slide horizontally, gently add Rinse Buffer to the slide drop wise to flood the tissue specimen.
3. Pour off the Rinse Buffer, and repeat steps 2 and 3 for a total of 5 times
4. Apply 4 drops of Rinse Buffer to the tissue and incubate for a minimum of 2 minutes
5. Pour off Rinse Buffer and tap the edge of each slide onto a paper towel.
6. Repeat

2.2.4 Staining Procedure

After the first iteration of rinsing, two drops of the Blocking Reagent (normal goat serum in phosphate buffered saline containing carrier protein) included in the CHEMICON kit are added to the specimen and incubate for 5 minutes. Blocking Reagent minimizes non-specific binding of antibodies, which can lead to false-positive results. The slide is rinsed once while holding it at a 45 degree angle for 15 seconds. Excess rinsing buffer is removed.

Hypoxyprome-1 MAb1 primary antibody conjugated with FITC is diluted to 1:50 or 1:100 concentration in antibody diluent, and 100 µl is added to the tissue. The slide is incubated for 10 minutes, and then rinsed according to the “Rinse Procedure” described above. Next, the secondary anti-FITC Mab antibody conjugated with HRP is diluted to a 1:50 concentration in antibody diluent and 100 µl are added to the tissue. The slide is once again rinsed according to the procedure.

Chromogen reagent is prepared by combining Chromogen A (3,3' Diaminobenzidine diluted in TBS) to Chromogen B (hydrogen peroxide diluted in TBS, containing Tween® 20) in a 1:25 ratio. Chromogen reagent contains a molecule that will precipitate brown to black when acted upon by HRP, to allow the visualization of the location of pimonidazole. Enough reagent is added to the tissue to sufficiently cover the area enclosed by the PAP Pen barrier. After a 10 minute incubation period, the slide is rinsed again.

In order to provide the contrast necessary in order to view hypoxia with cell histology, two drops of Hematoxylin Counter Stain solution (Mayer's hematoxylin counter stain) are added to the tissue, and incubated for 5 minutes. Hematoxylin stains cellular nuclei blue-violet, which allows co-visualization of cellular histology and hypoxic pimonidazole binding. Following incubation, the slide is rinsed according to procedure and placed into a Coplin jar filled with deionized water.

To preserve the tissue and staining, the tissue is dehydrated by dipping into Coplin jars in the following orders for the following durations:

1. 100% Ethanol 4 x 1 minute
2. Xylene 3 x 1 minutes

Three drops of a xylene-based adhesive are added to the slide to mount coverslips. Slides are allowed to dry overnight.

If the procedure is done correctly, positive controls containing a known region of hypoxia will stain brown in hypoxic regions and blue in non-hypoxic regions, whereas negative controls containing an irrelevant concentration-matched mouse IgG as a primary antibody will appear

completely blue. Necrotic regions, containing cells that have died in a non-apoptotic manner, will stain very lightly blue if necrosis is recent, or not at all if necrosis is old.

2.3 References:

1. Kleiter, M. M., Thrall, D. E., Malarkey, D. E., Ji, X., Lee, D. Y., Chou, S. C., and Raleigh, J. A. A comparison of oral and intravenous pimonidazole in canine tumors using intravenous CCI-103F as a control hypoxia marker. *Int J Radiat Oncol Biol Phys*, 64: 592-602, 2006.
2. Janssen, H. L., Haustermans, K. M., Sprong, D., Blommestijn, G., Hofland, I., Hoebbers, F. J., Blijweert, E., Raleigh, J. A., Semenza, G. L., Varia, M. A., Balm, A. J., van Velthuysen, M. L., Delaere, P., Sciot, R., and Begg, A. C. HIF-1alpha, pimonidazole, and iododeoxyuridine to estimate hypoxia and perfusion in human head-and-neck tumors. *Int J Radiat Oncol Biol Phys*, 54: 1537-1549, 2002.
3. Janssen, H. L., Haustermans, K. M., Sprong, D., Blommestijn, G., Hofland, I., Hoebbers, F. J., Blijweert, E., Raleigh, J. A., Semenza, G. L., Varia, M. A., Balm, A. J., van Velthuysen, M. L., Delaere, P., Sciot, R., and Begg, A. C. HIF-1alpha, pimonidazole, and iododeoxyuridine to estimate hypoxia and perfusion in human head-and-neck tumors. *Int J Radiat Oncol Biol Phys*, 54: 1537-1549, 2002.
4. Khanna, S., Roy, S., Maurer, M., Ratan, R., and Sen, C., Oxygen-Sensitive Reset Of Inducible Hif Transactivation Response: Prolyl Hydroxylases Tune The Biological Normoxic Setpoint. *Free Radic Biol Med*. 2006 June 15; 40(12): 2147–2154.
5. Immunoperoxidase Secondary Detection System. CHEMICON IHC Select®, August 2005. Temecula CA
6. Hypoxyprobe™-1 Plus Kit for the Detection of Tissue Hypoxia. CHEMICON International, 2006

CHAPTER THREE

ESTABLISHMENT OF A SUITABLY HYPOXIC *IN VIVO* TUMOR MODEL

3.1 Introduction

In order to test for new hypoxia-selective BNCT compounds, a suitable model must be established. To simulate the circulation and metabolism present in human BNCT patients, an *in vivo* mouse model was chosen for testing. The proper tumor cell line, mouse species, and proper tumor size must be selected in order to simulate the levels of hypoxia that normally exist in the human cancers that BNCT will be used to treat. Additionally, immunological rejection of tumors may occur if the tumor cell line is incompatible with its host mouse species.

A literature review reveals that the murine SCCVII squamous cell carcinoma line and EMT-6 murine mammary carcinoma are known to exhibit hypoxia (1) and form rapidly growing subcutaneous tumors in C3H and BALB/c mice, respectively, with tumor volume doubling times of approximately 4-7 days. The tumor size necessary to achieve hypoxia is approximately 10 mm in diameter for 10% and 20% hypoxia in SCCVII and EMT-6, respectively.

3.2 Hypoxic Tumor Model

3.2.1 Protocol for Inducing *In Vivo* Hypoxic Tumors

To induce SCCVII tumors in mice, SCCVII and EMT-6 cells must first be cultured and demonstrated to be viable *in vitro*. The cells are grown in T75 flasks. When the cells appear confluent, each flask contains 5×10^6 to 10×10^6 cells. The cells are trypsinized to detach them from each other and the bottom of the flask, and counted in a Coulter counter. The cells are gently centrifuged in Falcon F50 tubes to concentrate them without rupturing the cell membranes, and diluted in medium to concentration of 2×10^6 cells per 0.1 ml.

C3H female brown mice are anesthetized by brief inhalation exposure to isoflurane and inoculated subcutaneously onto their backs with 2.0×10^6 SCCVII cells. Alternatively, for EMT-6 tumors, 1.0×10^6 EMT-6 cells are used for inoculation. Half as many EMT-6 cells are required due to its faster growth time and increased risk of necrosis.

Infected mice are maintained according to standard animal protocol. Tumors are measured weekly for the first two weeks and daily thereafter until a suitable size is reached. Tumors must not exceed 10% body weight (about 1000 mg) in keeping with standards of humane treatment.

After the tumors reached about 1 cm in diameter, mice were given an intraperitoneal injection with 60 mg/kg of pimonidazole hydrochloride at a concentration of 34 mM or 10 g/L in 0.9% saline. The pimonidazole injection can also be delivered as a tail-vein IV injection or a retro-

orbital IV injection. Average amounts were 1.5 mg/mouse, or 0.15 mL of solution/mouse. After 90 minutes, corresponding to three blood plasma half-lives of pimonidazole hydrochloride in mice, the mice were sacrificed. Both tumors and livers from each mouse were harvested and fixed in formalin and stained according to the procedure describe in Chapter 2.

3.2.2 Results

Following staining, slides were viewed under a standard digital light microscope at various magnifications. Various sections of each tissue were viewed. Images were digitally enhanced to correct for the whitewashing that occurred from the microscope lamp.

In Figure 3.1, two different sections prepared from the same SCCVII tumor with hypoxic cells appearing brown in comparison to the blue hematoxylin stained cells in both 1:50 and 1:100 dilutions of primary antibody. The SCVII tumor grew to 9 x 11 mm in size in 16 days. The mouse was given the normal dosage of pimonidazole, which was administered via intraperitoneal injection. The similarities between hypoxic structures in Figure 3.1a and Figure 3.1b indicate consistency in pimonidazole binding and immunohistochemical staining. Figure 3.1c is a negative control using concentration matched irrelevant IgG mouse antibody, which demonstrates that non-specific binding is at an acceptably low amount.

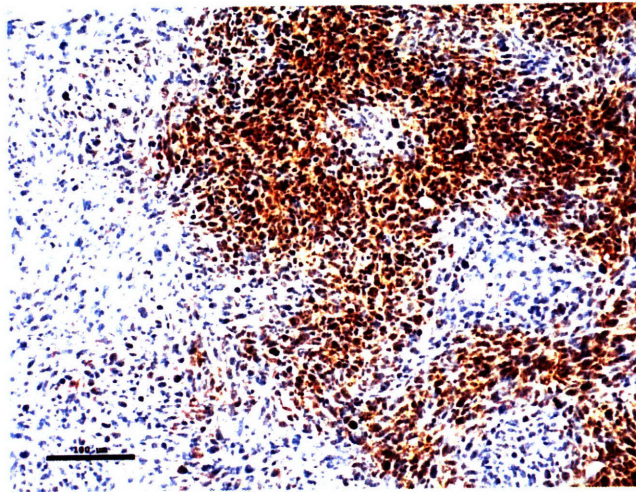


Figure 3.1a Hypoxia in SCCVII tumor grown to 9 x 11 mm. Primary antibody was diluted to 1:50. Brown-stained cells indicate regions of hypoxia, whereas blue-stained cells indicate non-hypoxia.

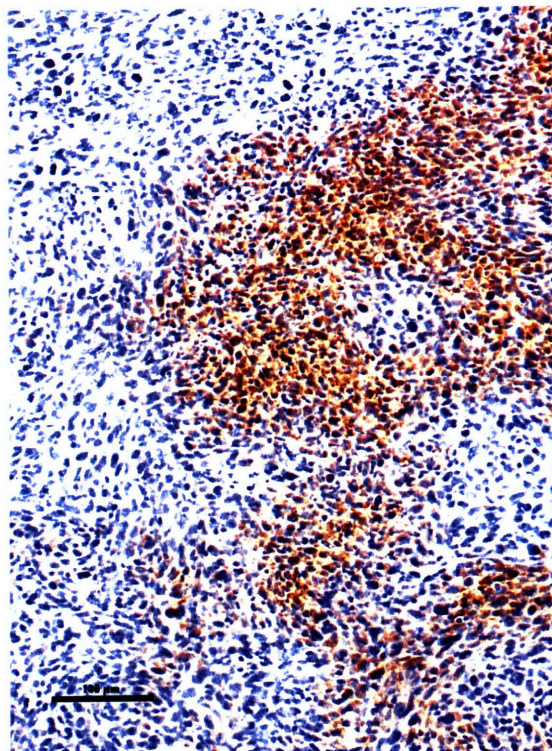


Figure 3.1b Hypoxia in SCCVII tumor grown to 9 x 11 mm. Primary antibody was diluted to 1:100. Brown-stained cells indicate regions of hypoxia, whereas blue-stained cells indicate non-hypoxia.

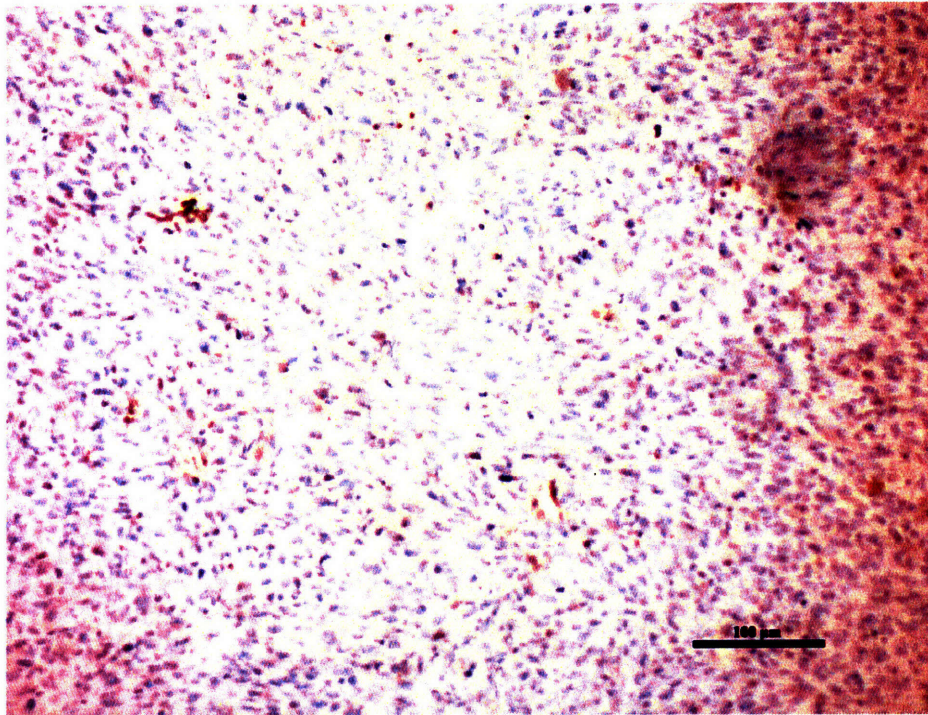


Figure 3.1c Negative control in SCCVII tumor. Blue staining indicates the antibody did not detect false positive non-specific binding.

To visualize the presence of tumor necrosis, another section of the slide was viewed. In Figure 3.2, the lack of color and cellular matter in the center of the brown hypoxic region is the result of tumor necrosis, which occurs when the absence of O_2 and nutrients leads to non-apoptotic cell death.

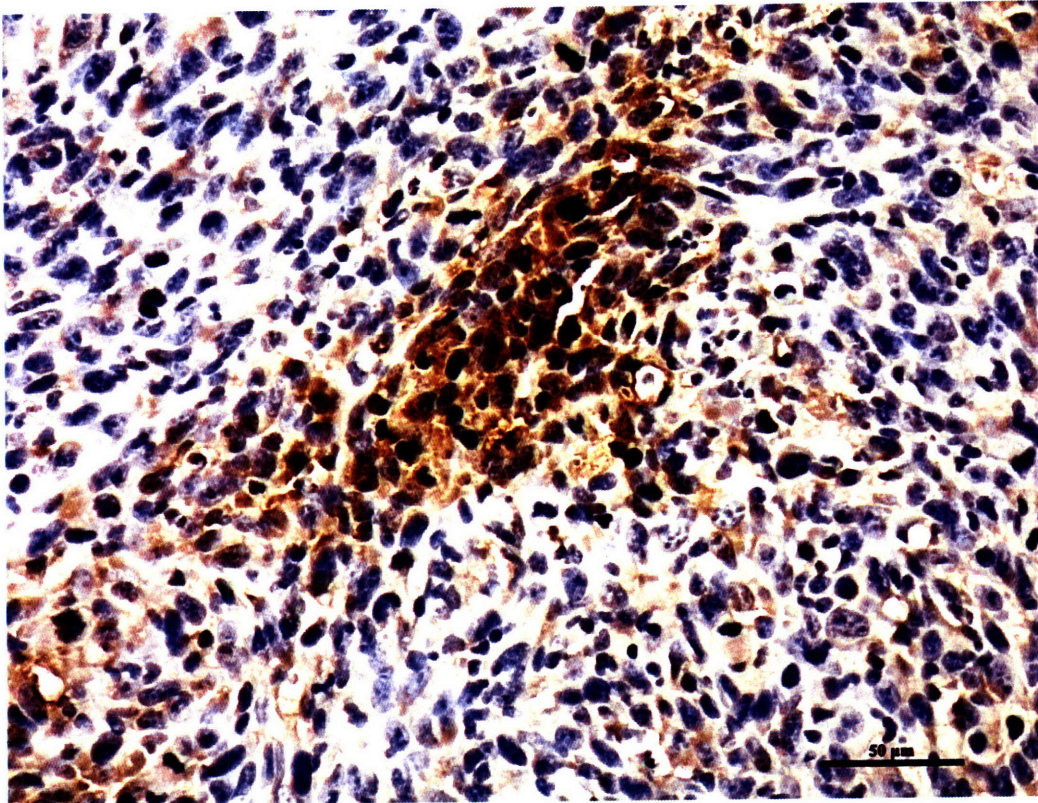


Figure 3.2 Necrosis in the center of an SCCVII tumor grown to 9 x 11 mm. Brown cells are hypoxic, blue cells are non-hypoxic, and the absence of color and cellular matter indicates necrosis.

Results for EMT-6 tumors in BALB/c nude mice demonstrate a more drastic gradient from non-hypoxic, to hypoxic, to necrotic regions of tumor. These results are characteristic of the faster growth of EMT-6 cells. In Figure 3.3, an EMT-6 tumor exposed to pimonidazole *in vitro* is shown after staining. The non-hypoxic cells in the bottom-left of the image transition to recent necrosis in the top-right of the image.

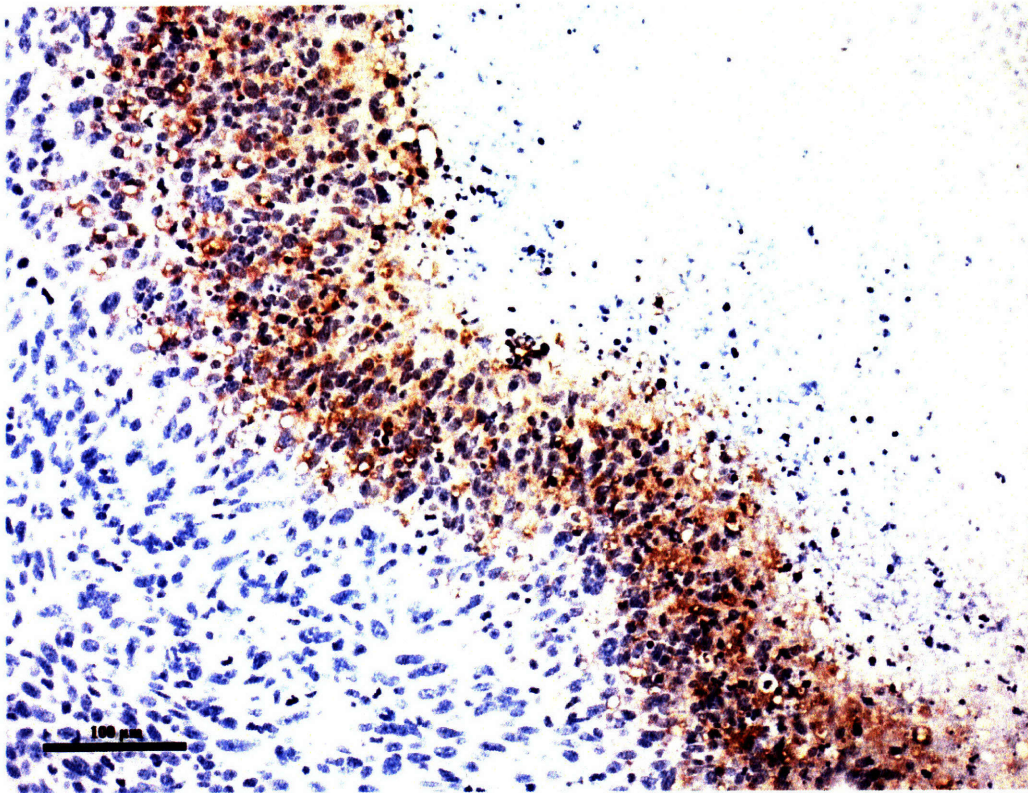


Figure 3.3 Hypoxic gradient in an EMT-6 tumor. A steep gradient occurs from the blue non-hypoxic cells in the bottom-left hand corner to the faded blue necrotic cells in the top-right hand corner.

A liver sample was also taken from the same pimonidazole-injected mouse and stained for hypoxia. Given that pimonidazole protein adducts form when $pO_2 < 10$ mmHg in tissue, and normal liver have cells at, or below this pO_2 , staining in liver tissue is expected (2). In Figure 3.4, the surface of the liver tissue is shown on the left. Approximately 400 μm from the surface, a steady level of hypoxia is evident, as demonstrated by constant staining visible from the middle to the right half of the image.

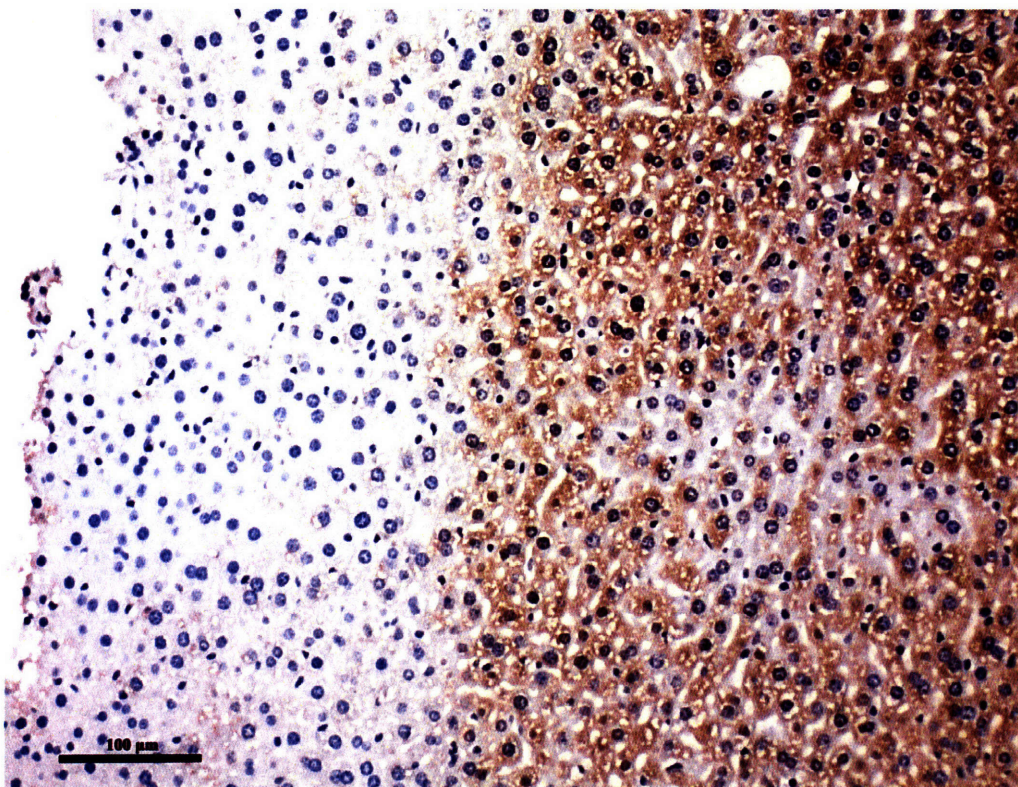


Figure 3.4 Hypoxic staining in normal liver. The liver was taken from the same mouse that was implanted with EMT-6 in Figure 3.3. The surface of the liver is on the left, whereas the hypoxic interior of the liver is visible by the brown staining on the right.

3.2.3 Conclusion

A comparison of the staining patterns visible in Figures 3.1 – 3.4 to published results indicates that the results obtained are consistent with proper pimonidazole chromogenic staining (3,4). Additionally, antibody binding is sufficient given that hypoxic staining patterns are consistent between serial sections of the same tissue in Figures 3.1a and 3.1b. Finally, comparison of SCCVII and EMT-6 tumor staining with published values indicates that the SCCVII and EMT-6 tumor model is suitably hypoxic for the testing of the binding of new boron-containing molecules (5,6).

3.3 References:

1. Rockwell, S., and Moulder, J.E. Hypoxic fraction of human tumors xenografted into mice: a review. *Int. J. Radiat. Oncol. Biol. Phys.* 1990;19:197-202.
2. Durand, R. E. and Raleigh, J. A. Identification of nonproliferating but viable hypoxic cells in vivo., *Cancer Res.* 58: 3547-3550, 1998.
3. Raleigh, J. A., Chou, S. C., Tables, L., Suchindran, S., Varia, M. A., and Horsman, M. R. Relationship of hypoxia to metallothionein expression in murine tumors. *Int J Radiat Oncol Biol Phys*, 42: 727-730, 1998
4. Raleigh, J. A., Chou, S. C., Calkins-Adams, D. P., Ballenger, C. A., Novotny, D. B., and Varia, M. A. A clinical study of hypoxia and metallothionein protein expression in squamous cell carcinomas. *Clin Cancer Res*, 6: 855-862, 2000
5. Samoszuk, M. and Corwin, M. A. Mast cell inhibitor cromolyn increases blood clotting and hypoxia in murine breast cancer. *Int J Cancer*, 107: 159-163, 2003
6. Olive, P.L., Luo, C-M., and Banáth, J.P., Local hypoxia is produced at sites of intratumour injection. *British Journal of Cancer* (2002), 429-435.
doi:10.1038/sj/bjc/6600059

CHAPTER FOUR

QUANTITATIVE MEASUREMENTS OF NEW COMPOUNDS

4.1 Introduction

The purpose of establishing an *in vivo* hypoxic tumor model was to test the localization of the new hypoxia-selective BNCT compounds being developed. In Chapter 4, a qualitative immunohistochemical assay was used to establish the presence of hypoxia in the SCCVII and EMT-6 mouse tumor models. The next task will be to quantitatively measure the concentration of boron in tumor tissue and healthy tissue when it is delivered as part of the new compound. The results will demonstrate whether the compound is suitable for further study in therapeutic trials. The two conditions that must be met are that the boron concentration meets a minimum required for BNCT, and that some selectivity is apparent in localization.

4.2 Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

4.2.1 Introduction to ICP-AES

ICP-AES is an analytical technique that can be used for the detection of boron concentration of samples in solution. ICP-AES works by using inductively coupled plasma to excite electrons in the sample and measuring the characteristic atomic spectra emitted in the process of electron

excitement. The relative levels of specific emission wavelengths are then used to calculate the concentration of the source atom. The advantage of ICP-AES is that low concentrations (<0.5 $\mu\text{g/g}$) are still detectable.

The preparation of a sample for ICP-AES requires that it is in liquid, particulate-free solution. Thus, tissue specimens weighing 25-50 mg are each digested at room temperature overnight in 0.15 ml of a 1:1 mixture of concentrated sulfuric acid and nitric acid. Prior to measurement, 0.5 ml of 10% Triton X-100 is added and the entire solution is diluted to 1.5 ml with water to result in a clear solution for analysis.

4.2.2 Results of ICP-AES Analysis of New Compound

After tumor induction, the mice were administered an IP injection of boronated pimonidazole dissolved in ethanol. Two mice received a dose of 66 mg/kg (compound A) and one other received a dose of 29 mg/kg (compound B). The mice were sacrificed approximately 90 minutes after injection and tissues were harvested for boron concentration analysis by ICP-AES. The results shown in Table 4.1 quantify the presence of boron in the different tissues with elevated levels in both liver and tumor as expected. Estimating that the tumors are 5% hypoxic (1) then measured increases of 3.1 $\mu\text{g/g}$ (formula 1) and 4.1 $\mu\text{g/g}$ (formula 7) in the gross boron concentration are significant and are equivalent to actual concentrations of 62 $\mu\text{g/g}$ and 82 $\mu\text{g/g}$ in the hypoxic regions.

Table 4.1 Boron concentration results from ICP-AES in different tissues from three mice administered two different doses of boronated pimonidazoles

Boron Concentration ($\mu\text{g/g}$)			
Tissue	Compound A (66 mg/kg)	Compound B (29 mg/kg)	Compound B (scaled to 66 mg/kg)
Tumor	7.2 ± 1.1	5.9	13.4
Muscle	4.1 ± 1.9	1.8	4.1
Brain	2.2 ± 0.6	0.7	1.6
Skin	3.7 ± 2.1	1.9	4.3
Blood	0.9 ± 0.1	2.7	6.2
Liver	11.9 ± 6.2	15.8	36

To obtain quantitative results, ten rats implanted with F98 glioma in their brains were administered a 100 mg/kg dose of the new compound and sacrificed at various time points of 45, 90, and 150 minutes. Results are shown in Figure 4.2. The tumors initially contained a five-fold greater concentration of boron. Though boron concentrations in the blood and brain remained relatively constant during the three time points, they dropped significantly in the tumor.

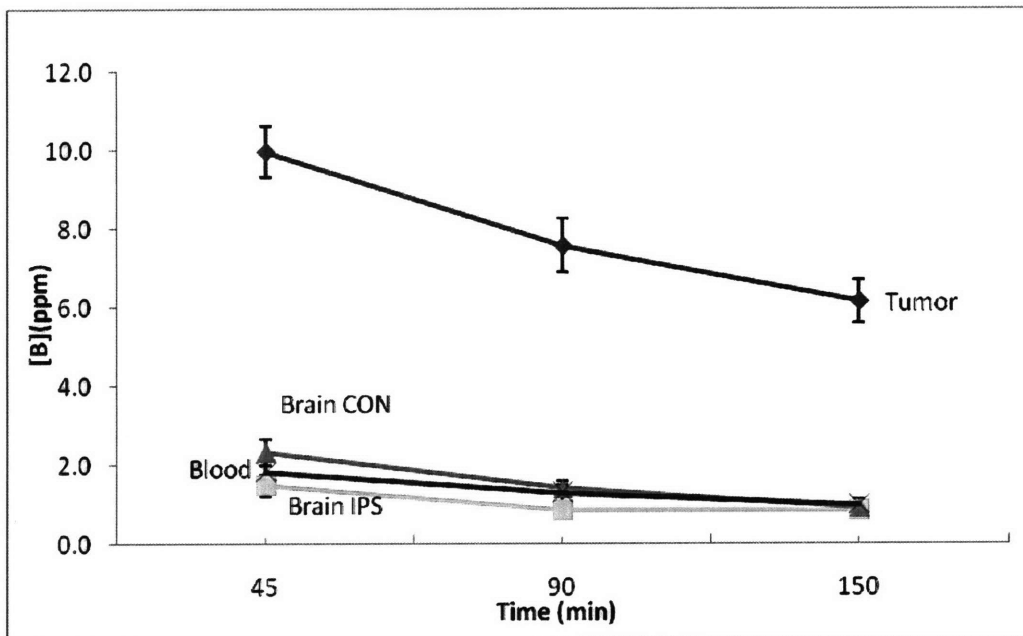


Figure 4.2 Boron concentrations in rat tissue injected with the new compound at 100 mg/kg. Concentration difference between F98 tumor and healthy tissue is greatest at 45 minutes.

4.2.3 Conclusion

To be effective as a therapy, the minimum delivered boron concentration in tumor must be 20-30 ppm (2). The results in Table 4.1 and Figure 4.2, adjusted to the fact that the compound localizes to hypoxic regions, and hypoxic regions comprise of approximately 5% of the tumor, demonstrate that boron concentration is well above the required concentration. If combined with the existing FDA-approved compound BPA-F, the new compound could prove to be a highly viable compound to deliver radiation dose selectively to tumor in a manner that is therapeutically effective.

4.3 References:

1. Rockwell, S., and Moulder, J.E. Hypoxic fraction of human tumors xenografted into mice: a review. *Int. J. Radiat. Oncol. Biol. Phys.* 1990;19:197-202.
2. M. Viaggi, M. A. Dargosa, J. Longhino, H. Blaumann, O. Calzetta, S. B. Kahl, G. J. Juvenal, M. A. Pisarev, Boron neutron capture therapy for undifferentiated thyroid carcinoma: preliminary results with the combined use of BPA and BOPP, *Applied Radiation and Isotopes* Volume 61, Issue 5, Topics in Neutron Capture Therapy: Proceedings of the Eleventh World Congress on Neutron Capture Therapy (ISNCT-11), November 2004, Pages 905-909.

CHAPTER FIVE

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS FOR FUTURE WORK

5.1 Introduction

In this concluding chapter, the work presented in this thesis is briefly reviewed. The immunohistochemical protocol for hypoxia visualization is briefly summarized. Further analysis and interpretation of results is briefly discussed. Finally, the thesis concludes with recommendations for future work and experiments.

5.2 Summary and Conclusions

This section summarizes the results of each major chapter.

5.2.1. Immunohistochemical Protocol for Hypoxia Visualization

Immunohistochemistry to measure and locate hypoxia is a qualitative technique that allows simultaneous visualization of histology and hypoxic cell distribution. The detailed procedures for producing high quality slides have been described. A number of improvements in the procedures were required in order to maximize contrast. Key improvements involved boiling the slides in citrate buffer for 10 minutes then allowing to cool at room temperature for 10 minutes,

using a PAP Pen, incubating each antibody for 10 minutes, and incubating with hematoxylin for 5 minutes. The result of these developments is a protocol that is more reliable, more accurate, and produces images superior in quality and clarity.

5.2.2 Establishment of a Suitably Hypoxic *In Vivo* Tumor Model

A hypoxic tumor model to measure boronated pimonidazole and other hypoxia-selective compounds has been established. Comparison of data to published results of hypoxia and immunohistochemical staining demonstrate that an animal model has been reproduced. The use of SCCVII, EMT-6, and F98 tumor cells in mice and rats will allow for easily reproducible and accepted methods of tumor hypoxia simulation, without the safety concerns associated with the use of human tumor cell lines. Furthermore, the relatively fast growth times of these tumors will allow for faster experimental turnaround.

5.2.3 Quantitative Measurements of New Compounds

The use of ICP-AES allows for a relatively quick and easy method of measure boron concentration for unenriched boron compound. Enriched compound containing only boron-10 is not necessary for studies. Values obtained for the concentration of the compound in both SCCVII and F98 tumors are promising. Though the experiments should be repeated to obtain more statistically sound data points, efficacy studies in animals should follow.

5.3 Recommendations for Future Work

This section presents recommendations for future work on research topics related to those discussed in this thesis.

5.3.1 Quantitative Analysis of Immunohistochemistry

Though the qualitative information conveyed in immunohistochemical staining for hypoxia is valuable, a complete quantitative evaluation would be valuable. Such a quantitative technique would involve mapping out the entire tissue section, not just what is visible in the field-of-view of the microscope and/or digital camera, and either manually or algorithmically calculated the ratio of brown hypoxia to total tissue.

5.3.2 Establishing Therapeutic Effectiveness

A crucial step in this project will be to run therapeutic experiments using boronated pimonidazole to demonstrate tumor growth recession. First, a system to image boronated pimonidazole must be developed. A primary antibody for the new compound is currently under development. Next, biodistribution studies with BPA and boronated pimonidazole should be performed to determine whether each compound affects the distribution of the other. Finally, for efficacy studies in animals, four comparison groups should be done with mice or rats that have tumors: nitroimidazole-carborane with neutrons, nitroimidazole-carborane and BPA with neutrons, BPA and neutrons, and neutrons only. Tumor size should be tracked and compared with known data with BPA only to check for improvement.