This final technical report is for our DOE grant DE-FG02-91ER61138 entitled "Subcellular boron and fluorine distributions with SIMS ion microscopy in BNCT and cancer research". This grant was in “NO Cost Extension Status” from 7/1/05 to 6/30/07. The development of a secondary ion mass spectrometry (SIMS) based technique of Ion Microscopy in boron neutron capture therapy (BNCT) was the main goal of this project, so that one can study the subcellular location of boron-10 atoms and their partitioning between the normal and cancerous tissue. This information is fundamental for the screening of boronated drugs appropriate for neutron capture therapy of cancer. Our studies at Cornell concentrated mainly on studies of glioblastoma multiforme (GBM). The early years of the grant were dedicated to the development of cryogenic methods and correlative microscopic approaches so that a reliable subcellular analysis of boron-10 atoms can be made with SIMS. In later years SIMS was applied to animal models and human tissues of GBM for studying the efficacy of potential boronated agents in BNCT. Under this grant the SIMS program at Cornell attained a new level of excellence and collaborative SIMS studies were published with leading BNCT researchers in the U.S. A list of peer-reviewed publications under this grant is provided below and a few highlights are discussed in this report.

SIMS studies in T98G human glioblastoma cells for understanding mechanistic aspects of boronophenylalanine (BPA) uptake in tumor cells

The BPA is still being used in experimental trials of GBM, melanoma, and liver metastases in some European and Asian countries. To understand why BPA selectively targets the cancer cells has long remained a challenging question in BPA-mediated BNCT. This is one important area of BNCT research where SIMS has played a pivotal role by making sub-cellular scale observations of boron-delivery from BPA in animal and cell culture models of GBM. The infiltrating tumor cells in the normal brain are the main targets of clinical BNCT. Since infiltrating tumor cells are protected by the blood-brain-barrier, boron-delivery to these cells can be significantly different from the cells in the main tumor mass where the blood-brain-barrier is compromised. The application of SIMS, in conjunction with cryogenic sampling, has the capability for the assessment of single cell boron measurements for resolving these complex issues of the efficacy of a particular boron containing drug in targeting the relevant tumor cells in GBM. A brief discussion of our SIMS observations on BPA is discussed below.

Our SIMS observations provided the first direct evidence in cryogenically prepared cells that BPA targets infiltrating tumor cells in the normal brain, also known as the satellite or clusters of malignant cells peppered in the normal brain far away from the main tumor mass (Smith et al., Cancer Res. 56: 4302-4306, 1996). This paper also indicated a main flaw in the ASSUMPTION that is made for radiation dosimetry calculations in clinical BNCT: the boron content of tumor cells in the main tumor mass is the same as the infiltrating tumor cells. Our SIMS observations indicated that the infiltrating tumor cells had approximately 50% of boron than found in tumor cells in the main tumor mass. These observations had critical implications in clinical BNCT. Further SIMS studies of BPA in animal (Smith et al., Cancer Research, 61: 8179-8187, 2001) and cell culture models of GBM (Chandra et al., Radiation Research, 157: 700-710, 2002) indicated that

a longer exposure of BPA can elevate the boron content of infiltrating tumor cells and provide a nearly 3:1 partitioning between the infiltrating tumor cells and the normal brain. Even though our SIMS observations were limited to cell culture and rat models of GBM, the 6 hr. long BPA exposure supported by our studies was clinically implemented in experimental trials of GBM in Sweden. However, the reasons behind this increased boron uptake at the longer 6 hr. BPA exposure (versus the shorter 1 and 2 h. exposures) remained unclear. To understand the mechanism of this increased uptake of 10B from BPA, we developed a SIMS approach for directly imaging the net entry of 13C15N isotopically labeled amino acids. By imaging the marker label at mass 28(13C15N) with SIMS, we were able to study the subcellular distribution of phenylalanine. Further extension of this study by using a direct comparison of 10B entry from BPA to the net entry of the isotopically labeled phenylalanine provided unique SIMS observations relevant to the mechanistic aspect of BPA uptake and metabolism in cells. First, there was a comparable increase in the labeled amino acid or in the 10B from BPA between the 2 and 6 hr. exposures of T98G glioblastoma cells. Second, the subcellular distribution of the 28CN label was different than 10B from BPA. The 28CN was distributed throughout the cell, but 10B was distinctly lower in the mitochondria-rich perinuclear region of T98G glioblastoma cells (as revealed by laser confocal microscopy imaging of rhodamine 123 fluorescence). These observations indicate that (i) the higher uptake of BPA and 28CN-labeled phenylalanine at 6 hr. vs. 2 hr plausibly represents a basic similarity between BPA and phenylalanine in a time-dependent entry mechanism through the plasma membrane in response to cellular requirements for the amino acid and (ii) intracellular processes recognize BPA as a different molecule than phenylalanine and the metabolism of BPA is distinctly different than that of phenylalanine. Further understanding of the higher accumulation of BPA with longer exposures was provided by the Cell Cycle preference of BPA uptake. By pulsing 5-bromodeoxyuridine with BPA, SIMS was able to provide the boron concentrations in individual cells in the DNA synthesizing phase (S-phase) of the cell cycle. These observations revealed elevated levels of boron from BPA in the S-phase cells. Therefore, the movement of the cell cycle through the S-phase will be at least one reason that would partially enhance the boron uptake of brain tumor cells with longer exposures of BPA. A major publication has been communicated on these observations (Chandra, S. and Lorey, D. R. SIMS ion microscopy imaging of boronophenylalanine and 13C15N-labeled phenylalanine in human glioblastoma cells: Relevance of subcellular scale observations to BPA-mediated boron neutron capture therapy of cancer. Int. J. Mass Spectrom. 260: 90-101, 2007).

SIMS studies of boron-delivery to mitotic cells

As many tumor cells can be found in the mitotic M phase of the cell cycle, the evaluation of their boron-targeting has always remained a question in BPA mediated BNCT. In a recent SIMS publication, we now show the subcellular scale boron distribution in metaphase cells. These evaluations included studies of two BNCT agents, BPA and N4. The N4 is a boron analogue of thymidine with characteristics of the S-phase targeting. Our SIMS observations in this study revealed that mitotic cells have different characteristics of boron uptake than interphase cells and should be evaluated for this difference for each boron compound in clinical BNCT (Chandra, S., Tjarks, W., Lorey,

D. R., and Barth, R. F. Quantitative subcellular imaging of boron compounds in individual mitotic and interphase human glioblastoma cells with SIMS. J. Microsc. 229: 92-103, 2008). The 3-D SIMS imaging methodology shown in this paper should be feasible to future screening of any boron containing agents in mitotic cells.

SIMS studies of GB-10 and BPA in hamster cheek pouch oral cancer model

In April 2005, the PI, Dr. Subhash Chandra visited Argentina to do collaborative research with Dr. Amanda Schwint of the National Atomic Energy Commission, Buenos Aires (April 11-19, 2005). This visit was supported by DOE's sister lab program. SIMS studies were deemed important for observing the distribution of boron from BPA and GB-10 in cancerous and normal tissues of the hamster cheek pouch. Initial SIMS observations in this collaboration revealed a consistent pattern of a higher accumulation of boron from BPA in the nuclei of cells in the cancerous tissue. The boron from GB-10 was observed to be distributed in low concentration throughout the tissue.

Other studies in partial support by the grant

In several publications this grant is acknowledged for the partial support. These publications represent diverse fields of fundamental cell biology and applied biomedical research with SIMS and in some cases extend the application of methods developed under this grant. A detailed study of cell division was completed in normal renal epithelial LLC-PK1 cells for understanding the location of calcium stores in all various phases of mammalian cell division (Chandra, S. Quantitative imaging of subcellular calcium stores in mammalian LLC-PK1 epithelial cells undergoing mitosis by SIMS ion microscopy. Eur. J. Cell Biol., 84: 783-797, 2005). What is remarkable about this work is that in comparison to the depletion of calcium stores observed in the spindle of metaphase human glioblastoma cells, revealed by SIMS in a previous study (Chandra, S. 3D subcellular SIMS imaging in cryogenically prepared single cells. Applied Surface Science, 231-232: 467-469, 2004), the normal cells show the presence of well aligned calcium stores in the spindle of metaphase cells. Taken together, SIMS observations in these two cell types provide valuable hints to the plausible alteration of calcium signaling in dividing glioblastoma cells.

A study of Gd isotope localization was completed with SIMS in the skin tissue of patients suffering from nephrogenic systemic fibrosis. SIMS is currently the only technique to reveal the presence of very low concentrations of soluble Gd associated with the skin tissue of patients treated with MRI contrast enhancing agents (Abraham et al., SIMS imaging of gadolinium isotopes in tissue from nephrogenic systemic fibrosis patients: Release of free Gd from MRI contrast agents. Appl. Surf. Sci., 2008).

In another unique study the SIMS methodology developed for 13C imaging under the current grant was used for single cell imaging of soil bacterial population for degradation of organic molecules (Chandra et al., Dynamic SIMS ion microscopy imaging of individual bacterial cells for studies of isotopically labeled molecules. Appl. Surf. Sci.

2008). This study has opened new avenues of SIMS research in microbiology for identification of soil bacterial cells involved with degradation of organic molecules.

The grant is acknowledged in a review article on cryogenic sample preparation for subcellular localization of elements and molecules with SIMS presented in a roundtable discussion at the XVI International Conference on Secondary Ion Mass Spectrometry (SIMS), held in Kanazawa, Japan, 2007 (Chandra, S. Challenges of biological sample preparation for SIMS imaging of elements and molecules at subcellular resolution. Appl. Surf. Sci., in press). The grant is also acknowledged in a recent study which shows that by using 5-bromouridine and 5-iododeoxyuridine both RNA distribution and DNA replication can be imaged in the same cell by SIMS (Chandra, S. Subcellular imaging of RNA distribution and DNA replication in single mammalian cells with SIMS: the localization of heat shock induced RNA in relation to the distribution of intranuclear bound calcium. J. Microsc. in press). These methodological developments further enhance the utility of SIMS for drug screening applications in BNCT and other areas of medical research.

Publications (Peer-reviewed) Under Grant:

Bennet, B. D., Zha, X., Gay, I., and Morrison, G. H. Intracellular boron localization and uptake in cell cultures using imaging secondary ion mass spectrometry for neutron capture therapy of cancer. Biol. Cell 74: 105-108 (1992).

Bennett, B.D., Mumford-Zisk, J., Coderre, J.A., and Morrison, G.H. Subcellular localization of *p*-boronophenylalanine-delivered boron-10 in the rat 9L gliosarcoma: Cryogenic preparation in vitro and in vivo. Radiation Res. 140: 72-78 (1994).

Chandra, S. and Morrison, G. H. Imaging of ion and molecular transport at subcellular resolution by secondary ion mass spectrometry. Int. J. Mass Spec. Ion. Proc. 143: 161-176 (1995).

Gay, I., Schinazi, R. F., Liotta, D. C., and Morrison, G. H. Subcellular localization of a boronated nucleoside using ion microscopy. In “Cancer: Neutron Capture Therapy”, proceedings of the 6th International Symposium on Neutron Capture Therapy for Cancer, held October 31-November 4, 1994, in Kobe, Japan. Yutaka Mishima (Ed.), Plenum Press, New York, 1996, pp. 209-213.

Smith, D. R., Chandra, S., Coderre, J. A., Wilson, J. L., Micca, P. L., Nawrocky, M. M., and Morrison, G. H. Ion microscopy imaging of boron from BNCT drugs in cryogenically prepared tissues. In “Cancer: Neutron Capture Therapy”, proceedings of the 6th International Symposium on Neutron Capture Therapy for Cancer, held October 31-November 4, 1994, in Kobe, Japan. Yutaka Mishima (Ed.), Plenum Press, New York, 1996, pp. 215-220.

Smith, D. R., Chandra, S., Coderre, J. A. and Morrison, G. H. Ion microscopy imaging of ^{10}B from p-boronophenylalanine in a brain tumor model for boron neutron capture therapy. Cancer Res. 56: 4302-4306 (1996).

Smith, D. R., Chandra, S., Coderre, J. A., Barth, R. F., Yang, W., Liu, L., Wilson, J. L., Micca, P. L., Nawrocky, M. M., Rotaru, J. H., and Morrison, G. H. Quantitative imaging of boron-10 in rat brain tumor models for BNCT. In "Advances in Neutron Capture Therapy", Volume 2, Chemistry and Biology, B. Larsson, J. Crawford and R. Weinreich, Eds. Elsevior, Amsterdam, 1997, pp. 308-314.

Chandra, S., Lorey II, D. R. Lessig, S. L., Smith, D. R., and Morrison, G. H. Quantitative imaging of boron from BPA and BSH in glioblastoma cells with ion microscopy. In "Advances in Neutron Capture Therapy", Volume 2, Chemistry and Biology, B. Larsson, J. Crawford and R. Weinreich, Eds. Elsevior, Amsterdam, 1997, pp. 315-320.

Chandra, S., Smith, D. R. and Morrison, G. H. Subcellular imaging by dynamic SIMS ion microscopy. Analyt. Chem. 72: 104A-114A (2000).

Morris, G. M., Smith, D. R., Patel, H., Chandra, S., Morrison, G. H., Hopewell, J. W., Rezvani, M., Micca, P. L., and Coderre J. A. Boron microlocalisation in oral mucosal tissue: Implications for boron neutron capture therapy. Brit. J. Cancer, 82: 1764-1771 (2000).

Chandra, S. and Lorey, II, D.R. Feasibility of SIMS ion microscopy for cancer research: Single cell isotopic imaging for chemical composition, cytotoxicity, and cell cycle recognition. Cell. Mol. Biol. (Noise-Le-Grand) 47: 503-518 (2001).

Lorey, II, D. R., Chandra, S., Smith, D. R., and Morrison, G. H. Quantitative analysis of boron in co-cultures of human glioblastoma T98G cells and normal cell lines within the same field of view of ion microscopy imaging. In "Frontiers in neutron capture therapy", Proceedings of the 8th International Symposium on Neutron Capture Therapy for Cancer, held September 13-18, 1998. M. F. Hawthorne, K. Shelly, and R. J. Wiersema (eds.). Kluwer Academic/Plenum Publishers, New York, 2001, pp. 887-891.

Smith, D. R., Joel, D. D., Coderre, J. A., Morris, G. M., Chandra, S., Nawrocky, M. M., Micca, P. L., Patel, H., and Morrison, G. H. Ion microscopy imaging of boron in animal tissue sections for neutron capture therapy. In "Frontiers in neutron capture therapy", Proceedings of the 8th International Symposium on Neutron Capture Therapy for Cancer, held September 13-18, 1998. M. F. Hawthorne, K. Shelly, and R. J. Wiersema (eds.). Kluwer Academic/Plenum Publishers, New York, 2001, pp. 893-897.

Smith, D. R., Chandra, S., Coderre, J. A., Joel, D. D., Slatkin, D. N., Chanana, A. D., Elowitz, E. H., Nawrocky, M. M., Micca, P. L., and Morrison, G. H. Ion microscopy imaging of boron from boronophenylalanine in surgically acquired samples of human brain tumor tissue. In "Frontiers in neutron capture therapy", Proceedings of the 8th International Symposium on Neutron Capture Therapy for Cancer, held September 13-18,

1998. M. F. Hawthorne, K. Shelly, and R. J. Wiersema (eds.). Kluwer Academic/Plenum Publishers, New York, 2001, pp. 899-903.

Chandra, S., Lorey, II, D. R., Smith, D. R., and Morrison, G. H. In vitro exposure of human T98G glioblastoma cells to mixed BNCT drugs: Acquiring independent quantitative images of boron atoms delivered by 10BPA and 11BSH in the same cell by SIMS ion microscopy. In "Frontiers in neutron capture therapy", Proceedings of the 8th International Symposium on Neutron Capture Therapy for Cancer, held September 13-18, 1998. M. F. Hawthorne, K. Shelly, and R. J. Wiersema (eds.). Kluwer Academic/Plenum Publishers, New York, 2001, pp. 905-909.

Gay, I., Lorey, II, D.R, Schinazi, R.F., Morrison, G.H., and Chandra, S. Dynamic SIMS ion microscopy imaging of intracellular boron accumulation from carboranyl nucleosides in glioma cells. Anticancer Res. 21: 2369-2376 (2001).

Lorey, II, D.R., Morrison, G.H., and Chandra, S. Dynamic SIMS analysis of boron from BNCT drugs in co-cultures: Single cell imaging of two different cell types within the same ion microscopy field of imaging. Analyt. Chem. 73: 3947-3953 (2001).

Smith, D.R., Chandra, S., Barth, R.F., Yang, W., Joel, D.D., and Coderre, J.A. Quantitative imaging and microlocalization of boron-10 in brain tumors and infiltrating tumor cells by SIMS ion microscopy: relevance to neutron capture therapy. Cancer Res. 61: 8179-8187 (2001).

Tjarks, W., Wang, J., Chandra, S., Ji, W., Zhuo, J., Lunato, A. J., Boyer, C., Li, Q., Usova, E.V., Eriksson. S., Morrison, G. H. and Cosquer, G. Y. Synthesis and biological evaluation of boronated nucleosides for boron neutron capture therapy (BNCT) of cancer. Nucleosides Nucleotides & Nucleic Acids 20: 695-698 (2001).

Chandra, S. and Smith, D. R., Organelle level chemical imaging of single cells by SIMS ion microscopy: relevance to NCT. In "Research and Development in Neutron Capture Therapy", W. Sauerwein, R. Moss, and A. Wittig, eds. Monduzzi Editore, via Ferrarese, Bologna, Italy, 2002, pp. 903-907.

Smith, D. R. and Chandra, S. Ion microscopy imaging of boron-10 in brain tumors, infiltrating tumor cells and other tissues. In "Research and Development in Neutron Capture Therapy", W. Sauerwein, R. Moss, and A. Wittig, eds. Monduzzi Editore, via Ferrarese, Bologna, Italy, 2002, pp. 919-923.

Chandra, S., Lorey, II, D.R. and Smith, D. Quantitative subcellular dynamic SIMS imaging of boron-10 and boron-11 isotopes in the same cell delivered by two combined BNCT drugs: In vitro studies on human glioblastoma T98G cells. Rad. Res. 157: 700-710 (2002).

Chandra, S., Kabalka, G.W., Lorey, II, D.R., Smith, D.R. and Coderre, J.A. Imaging of fluorine and boron from fluorinated-boronophenylalanine in the same cell at organelle

resolution by correlative SIMS ion microscopy and confocal laser scanning microscopy. Clin. Cancer Res. 8: 2675-2683 (2002).

Chandra, S. SIMS ion microscopy as a novel, practical tool for subcellular chemical imaging in cancer research. Appl. Surf. Sci. 203-204: 679-683 (2003).

Smith, D. R., Lorey, II, D. R. and Chandra, S. Subcellular SIMS imaging of gadolinium isotopes in human glioblastoma cells treated with a gadolinium containing MRI agent. Appl. Surf. Sci. 231-232: 457-461 (2004).

Chandra, S. Subcellular SIMS imaging of isotopically labeled amino acids in cryogenically prepared cells. Appl. Surf. Sci. 231-232: 462-466 (2004).

Chandra, S. 3D Subcellular SIMS imaging in cryogenically prepared single cells. Appl. Surf. Sci. 231-232: 467-469 (2004).

Barth, R. F., Yang, W., Al-Madhoun, A. S., Johnsamuel, J., Byun, Y., Chandra, S., Smith, D. R., Tjarks, W., and Eriksson, S. Boron containing nucleosides as potential delivery agents for neutron capture therapy of brain tumors. Cancer Res. 64: 6287-6295 (2004).

Chandra, S. Quantitative imaging of subcellular calcium stores in mammalian LLC-PK1 epithelial cells undergoing mitosis by SIMS ion microscopy. Eur. J. Cell Biol., 84: 783-797 (2005).

Chandra, S. and Lorey, II, D. R. SIMS ion microscopy imaging of boronophenylalanine (BPA) and 13C15N-labeled phenylalanine in human glioblastoma cells: Relevance of subcellular scale observations to BPA-mediated boron neutron capture therapy of cancer. Int. J. Mass Spectrom, 260: 90-101 (2007).

Chandra, S., Tjarks, W., Lorey, II, D. R., and Barth, R. F. Quantitative subcellular imaging of boron compounds in individual mitotic and interphase human glioblastoma cells with imaging secondary ion mass spectrometry (SIMS). J. Microsc. (Oxford) 229: 92-103 (2008).

Chandra, S. Challenges of biological sample preparation for SIMS imaging of elements and molecules at subcellular resolution. Appl. Surf. Sci. DOI: 10.1016/j.apsusc.2008.05.073

Chandra, S., Pumphrey, G., Abraham, J. M., and Madsen, E. L. Dynamic SIMS ion microscopy imaging of individual bacterial cells for studies of isotopically labeled molecules. Appl. Surf. Sci. DOI: 10.1016/j.apsusc.2008.05.129

Abraham, J. L., Chandra, S., Thakral, C., and Abraham, J. M. SIMS imaging of gadolinium isotopes in tissue from nephrogenic systemic fibrosis patients: Release of free Gd from MRI contrast agents. Appl. Surf. Sci., DOI: 10.1016/j.apsusc.2008.05.140

Chandra, S. Subcellular imaging of RNA distribution and DNA replication in single mammalian cells with SIMS: the localization of heat shock induced RNA in relation to the distribution of intranuclear bound calcium. J. Microsc. (Oxford), in press