Methods for Determining Metal Uptake in Cellular DNA for Auger Electron Therapy

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Abstract: Stable indium-labeled tetra(4-N-methylpyridyl)porphyrin [InTMPyP(4)] was evaluated as a carrier of a high Z atom, indium (In), into tumor cell DNA for its subsequent activation by radiation in a proposed radiotherapeutic technique, Auger
Electron Therapy (AET). Porphyrins with metals can bind to DNA and are useful vehicles for transporting the indium to the DNA of the tumor. AET combines the use of a metalloporphyrin with a stable high Z atom, such as indium, and photons emitted from radioactive brachytherapy seeds, such as iodine-125, to increase the radiation dose in the DNA of the tumor by generating a photoelectric effect in the K absorption edge of the indium (In) atom. This results in the emission of cascading Auger electrons that act as high LET radiation and thus impart significant non-reparable damage to the tumor compared to the radiation alone. The K absorption edge of In is 27.9 keV and the average photon energy of the iodine-125 seeds is ~ 28 keV.

Introduction and background: Porphyrin molecules have been reported as having an affinity for malignant tumor cells. They are readily metallated, and cationic porphyrins have been shown to bind DNA (1). The studies suggested the excellent potential of porphyrins for delivering high Z atoms metal atoms in or in close juxtaposition to cellular DNA where they can serve as one of the required agents for AET. Activation of the metal could result in Auger electron emission and DNA double strand breakage. Investigators have shown that significant biological damage results from Auger electron emission (2,3). Feinendegen, for example, predicted their value in "macromolecular surgery", when describing their biological toxicity (4). The two basic requirements for effecting this new treatment modality are, a) the availability of radiation sources whose energies are capable of activating the high Z atom; and b) the assurance that the high Z atom is localized at the DNA level so that the released Auger electrons can densely ionize the DNA. This work describes the experimental studies that were carried out to demonstrate the site of localization of either, an indium-labeled porphyrin, InTMPyP(4), or the In atoms transported by the porphyrin. InTMPyP(4) was postulated as a potential biomolecular carrier of indium into tumor cell DNA for use in Auger Electron Therapy (AET).

Materials and Methods: Confocal Laser Microscopy Studies. B16 murine melanoma cells with and without InTMPyP(4) [0.1 mg/ml] (Mid-Century Chemicals) were grown on glass slides in tissue culture plates in complete DMEM supplemented with 10% fetal bovine serum (HyClone), L-glutamine (2 μM) and penn-strep-fungisone. Cells were incubated for 24 hrs, the slides removed from the Petri dishes, washed thoroughly with PBS, fixed to the slides using ETOH, and coverslipped. The slides were scanned with laser light at a wavelength of 458 nm. Amber light conditions prevailed during the experiment to reduce undesired photosensitization

ICP-MS Studies i*n vitro* protocol. In-TMPyP(4) was dissolved in complete medium at concentrations ranging between 0.1 mg/ml and 0.2 mg/ml. 10^6 B16 cells were seeded in 100 mm Petri dishes with regular complete medium, grown for 24 hours, the medium aspirated and replaced with medium with and without InTMPyP(4). After 24 hours, cells were trypsinized, harvested, counted, fractionated with TCA, digested in nitric acid at 80°C and sent for ICP-MS measurements of indium uptake. All experiments were repeated twice.

ICP-MS Studies i*n vivo* protocol. B16 cells growing on monolayer were harvested and pelletized. The supernatant was removed and sterile saline was added to the cells to a final concentration of $3.5 \cdot 10^5$ cells per 50 µl. The food pad of each C57/BL mice mouse was sterilized with ETOH, and an aliquot of 50 µl was injected into the epidermis of the food pad. Three to four weeks after tumor implantation, a dosage of 40 mg/kg InTMPyP(4) in PBS was administered intraperitoneally (i.p.) as a single bolus injection of 200 µl. The clearance interval for the drug was varied over time to determine the highest tumor to normal tissue ratio of In atoms. Blood was obtained using an orbital draw, and mice were then euthanized. The heart (representing muscle), brain, spleen, liver, lungs and kidneys were dissected. Both hind feet were removed from the mouse, digested and sent for ICP-MS analysis along with the other tissues. The foot without tumor was used to provide the weight of the tumor by subtraction. Tissues were digested in nitric acid similar to that described above and sent for ICP-MS analysis.

Ethidium Bromide (EB) Displacement Assay. This assay was used to demonstrate the binding affinity of InTMPyP(4) for DNA. It is based upon competition at the DNA binding sites between ethidium bromide and the porphyrin. Into 2.8 ml of buffer solution (2mM of HEPES , 8 mM NaCl , 0.05 mM EDTA with a final Ph=7.0), 10 µl of 1.6µM EB and 10 µg calf thymus DNA was added. The spectrofluorimetric fluorescence intensity of this solution was taken as 100% at 598nm. InTMPyP(4), concentration 1µg /µl, was added to the solution in aliquots of 10 µl, allowing 2 minute intervals between readings to achieve equilibrium.

Results: *Confocal Laser Microscopy* showed ubiquitous fluorescence throughout the cell incubated with InTMPyP(4) including the nuclear area. No fluorescence was observed in the nucleus of cells without InTMPyP(4). *In vitro ICP-MS measurements* of the DNA-containing fraction of B16 cells with InTMPyP(4) were proportional to concentration, approaching 10^9 In atoms per cell at 0.2mg/ml InTMPyP(4), and were consistently a factor of 2 higher than the measurement in the cell fraction that did not contain the DNA. *In vivo ICP-MS measurements* of B16 murine melanoma tumors and normal organs and tissues, after the systemic administration of 40 mg/kg InTMPyP(4), showed the highest tumor-to normal tissue ratio at 5 hours after injection, with ~10^8 In atoms in the DNA-containing fraction. Kidney uptake of In atoms was always higher than that in other organs, which is consistent with the rapidly falling levels of In in blood. Uptake in 6 mice is shown in below in Figs. 1 and 2.

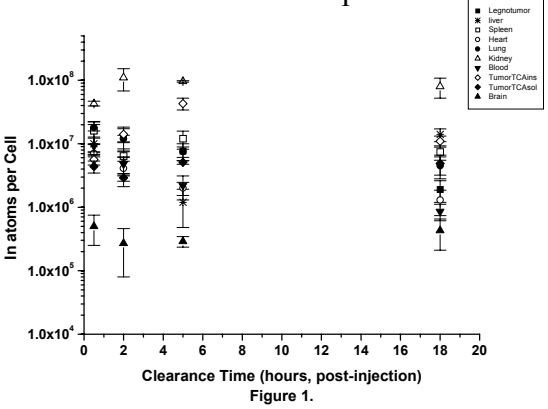


Figure 1.

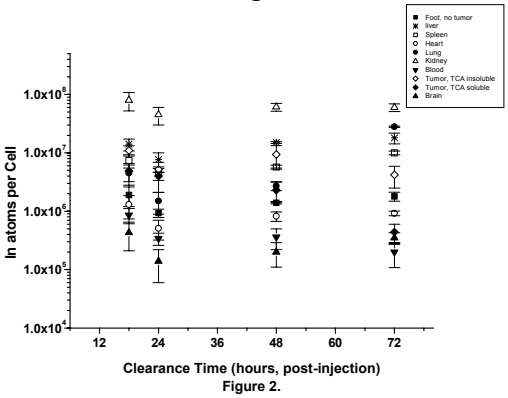
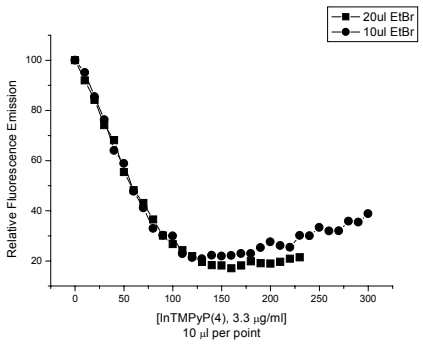


Figure 2.

The EB displacement assay also showed a strong and preferential binding of InTMPyP(4) to DNA, displaying linearity up to a 90% reduction in fluorescence intensity.



Discussion: Because AET relies heavily on the emission of the low energy, short-range Auger electrons in or in close proximity to DNA in order to effect DNA strand breakage, it was important to verify that the In atoms are, in fact, bound to DNA. The results of all assays carried out validate that the InTMPyP(4) carrier porphyrin does transport and bind In atoms to DNA. For clinical application as a radiation treatment,
soft energy X-rays above the K absorption edge of In (27.9 keV) are needed to generate a photoelectric effect in the In atoms. These can be provided by iodine-125 brachytherapy seeds (average energy = 28 keV) implanted directly into tumors using brachytherapy techniques. Brachytherapy is increasingly becoming a preferential treatment option for prostate cancer. The direct placement of the radiation sources in the tumor spares normal tissues within and around the treatment volume considerably. However, the major problem associated with using radiation to kill cancer cells is not addressed. The DNA of the cell must receive significant damage to cause cell lethality. But, the DNA mass is 0.25% that of the cell. The release of Auger electrons from In atoms bound tightly to tumor cell DNA can increase the biological damage to this critical site of the cell and increase the effective radiation dose delivered by iodine-125 seeds. AET studies in mice combining InTMPyP(4) with iodine-125 seeds are planned for the future. Results of all assays will be presented.

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