Intracerebral Diffusion of New Cholesterol-Based Anticancer Conjugate in Tumor-Bearing Rat Model

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ABSTRACT

Purpose: To study the diffusion and affinity in brain of a new cholesterol-based boron anticancer conjugate (cholesteryl 1,12-dicarba-*closo*-dodecaborane 1- carboxylate, BCH) using a tumor-bearing rat model and to examine the stability of BCH in the presence of cholesterol esterase and lipoprotein lipase.

Methods: The tumor-bearing rat model was produced by intracerebral implantation of rat 9L glioma cells. BCH was formulated in liposomes to enhance the aqueous solubility due to its extremely hydrophobic nature. Ten µl of BCH liposomes containing 150 µg BCH/mL was administrated to tumor-bearing rats intracerebrally and the BCH concentrations in tumor and normal tissues were examined at different time points. The degradation profiles of BCH and the control, cholesterol oleate, in cholesterol esterase, lipoprotein lipase were investigated.

Results: BCH concentrations in the tumor tissue at 2, 6, 8, and 14 hours after the intracerebral administration were 27.70, 12.51, 9.13, and 5.47 µg BCH/gm of tissue, respectively. The ratio of BCH between tumor tissue and normal tissue at 2, 6, 8, and 14 hours were 5.46, 1.56, 1.12, and 1.01, respectively. The affinity study showed that BCH concentration in tumor tissues was 4.45 µg/gm of tissue and in the normal tissue was 3.91 µg /gm of tissue, when both samples were taken 2 mm away from the BCH injection site in the opposite direction. Compared with the control, BCH had good stability in the presence of cholesterol esterase and lipoprotein lipase.

Conclusion: BCH formulated in liposomes distributed from the site of intracerebral injection rapidly. The adequate radiation time window appeared to be 2 hours or less. A small but statistically insignificant difference was found between the BCH concentrations in

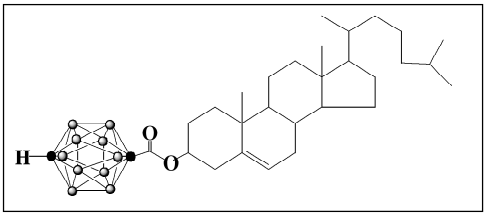

Figure 1. Cholesteryl 1,12-dicarba-closo-dodecarborane 1-carboxylate (BCH).

tumor tissue and normal tissue, both sampled with the same distance to the BCH injection site. BCH was stable in presence of cholesterol esterase and lipoprotein lipase, when compared with cholesterol oleate as the control.

INTRODUCTION

Each year more than 17,000 people in the United States are diagnosed with brain tumors.[1] The prognosis for brain tumor is poor due to the lack of effective treatment options. For the chemotherapeutic option, it is critical to deliver a sufficient amount of therapeutic agents to the brain tumor site. However, delivery of therapeutic agents to brain is technically challenging due to presence of blood brain barrier (BBB).[2] Many attempts of special drug delivery have been made to overcome this obstacle. One approach is to intracerebrally administer the agents within brain parenchyma through local delivery to tumor tissue.[3] The therapeutic molecules then diffuse from the site of administration to nearby cancer cells. This process offers the advantage of having high drug concentrations at the tumor site with limited exposure to normal tissues and organs in the rest of the body. Recently, our laboratory has developed a cholesterol-based anticancer agent containing carborane as the anticancer unit for boron neutron capture therapy. The new compound (Figure 1), cholesteryl 1,12-dicarba-*closo*-dodecaborane 1-carboxylate (BCH) mimics the native cholesteryl ester in structure and was found to be effectively taken up by brain glioma cells in cell culture studies.[4,5] The intracerebral administration of BCH in animal models, however, had not been studied.

Therefore, the purpose of the present study is to investigate the in vivo diffusion and tissue affinity of BCH in the brain after intracerebral BCH administration using a tumor-bearing rat model. The ratio of BCH concentrations in tumor versus surrounding normal tissue at different time points is an important factor for determination of the neutron radiation time window and it was investigated after intracerebral BCH administration. BCH stability in brain hydrolytic enzymes, including cholesterol esterase (CE) and lipoprotein lipase (LPL), was also examined.

EXPERIMENTAL SECTION

Materials

BCH was synthesized and purified according to a previously published method.[6] Cholesterol Dipalmitoyl DL-α-phosphotidylcholine (DPPC), CE, and LPL were purchased from Sigma Chemical Co. (St.Louis, Mo). All other chemicals were analytical grade.

Preparation of BCH Liposomal Formulation

Similar to native cholesteryl esters, BCH is extremely hydrophobic and thus, was formulated in liposomal formulation to enhance its aqueous solubility. The BCH liposomal formulation was prepared by thin film hydration method as described by Alanazi et al.[7] Briefly, cholesterol, phospholipid and BCH were dissolved in a 2:1 mixture of chloroform and methanol in a round bottom flask. The BCH to lipid ratio was 1:50 (w/w) and the total lipid (cholesterol and phospholipid) to water ratio was 1:38 (w/w). Cholesterol and phospholipid ratio was 0.33:1 molar ratio.[7] The solvents were evaporated under reduced pressure and a thin film of lipid was formed on the

bottom of the flask. The film was hydrated at a temperature above the phase transition temperature of the lipids with deionized water and glass beads (5 mm diameter) were added to ease the dissolution. The hydration process was carried out for several hours using a shaking water bath. Liposomes were separated from the glass beads by filtration through a Buchner funnel. The size of the liposomes was reduced using Emulsiflex B3 (Avestin, Ottawa, Canada) and measured at 25°C using a Nicomp Submicron Particle Sizer (Model 380, Nicomp, Calif).

Tumor-Bearing Rat Model

Male Fisher rats weighing about 250 g were used throughout the experiment. Rats were kept in standard cages (2 rats per cage) without restriction to water and food during the experiment. Rat 9L glioma cell line was used to introduce brain tumor in the animal. The cells were propagated in Dulbecco's Modified Eagle's Medium (DMEM, Fisher, Pittsburgh, Pa) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, Mo), 100 µg/mL streptomycin, and 100 U/mL of penicillin (Sigma, St. Louis, Mo) at 37°C and 5% CO_2 in 150 mm^2 flasks (Corning, Corning, NY). When the cells reached confluence, they were washed with DMEM, harvested using trypsin, centrifuged, re-suspended with phosphate buffer, counted, and kept in an ice bath for immediate use. The animals were anesthetized intramuscularly with ketamine (40 mg/kg) and xylazine (2.7 mg/kg). The rat scalps were shaved and sterilized with 70% ethyl alcohol. The rats were placed on a stereotaxic apparatus (Stoelting, Wood Date, Ill) and a middle incision was performed. The skull was exposed and a small burr hole was made 1 mm anterior to Lambdoid suture and 3 mm lateral (right) to Sagittal suture.[8] Four ml of the rat 9 L glioma cell suspension (4×10^4 cells) was injected intracerebrally 5 mm deep from the surface of the skull using a Hamilton syringe. The injection was performed over a period of 2 minutes and the needle was withdrawn slowly to minimize the backflow of cells in the needle track. The hole was filled with bone wax and the wound was closed with running silk sutures. The animals were watched over until they recovered from anesthesia and then returned to their cages.

Histological Verification of Tumor-Bearing Rat Model

To validate the tumor-bearing rat model, after 14 days of post-tumor implantation, brain tissues were obtained, fixed immediately with 10% buffered formalin, and sliced into coronal sections (3 to 5 mm in thickness). Tissue slides were placed in a paraffin tank, consolidated into a single unit using a Shandon HistoCenter 2 and cut into 4 µm sections using a Finesse Rotary Microtome (Thermo Shandon Inc, Pittsburgh, Pa). Micrometer sections were placed on a glass slide and subjected to washing with 70% to 100% of ethanol and 100% xylene to dehydrate sample and to remove the paraffin layer. The samples were stained with Hematoxylin and Eosin, using a robotic staining system (Leica Autostainer XL, Lecia Microsystems Inc, Bannockburn, Ill), and examined under light microscopy.

Diffusion and Affinity Studies After Intracerebral BCH Administration

The rats were ready for BCH diffusion studies when the tumor reached a relatively large size (about 14 to 16 days after implantation of tumor cells). The animals were injected intracerebrally with BCH liposomal formulation at the tumor site using the same procedure as described above for tumor implantation. Ten µl of BCH liposomal formulation (150 µg BCH/mL) was administrated for

each rat. To study BCH diffusion from the tumor site, 4 groups of rats (n = 3 for each time point) were sacrificed at 2, 6, 8, and 14 hours post-administration. Tumor tissue was carefully separated from the surrounding normal tissue and both tissue samples were prepared. In order to study BCH affinity to tumor and normal tissues in vivo, BCH liposomal formulation was injected intracerebrally 2 mm away from the tumor site. Rats (n =3) were sacrificed at 18 hours post-administration. A tumor tissue sample and a normal tissue sample were taken in opposite directions and both sampling sites were equidistant from the BCH administration site. All tissue samples were homogenized, digested in a Teflon-lined acid bomb, and quantitatively analyzed for BCH concentration using ICP-MS (inductively coupled plasma-mass spectrometry). ICP-MS provided a more sensitive method for analyzing the low BCH concentrations in tissue samples.

BCH Stability in Cholesterol Esterase and Lipoprotein Lipase

Ten mg of CE or 4 mg LPL were dissolved in 7.5 mL of phosphate buffered saline (pH 7.4). The concentrations of both enzymes were determined based on the recommendation of the supplier (Sigma). BCH liposomal formulation (0.673 µmole of BCH) was added and the mixture incubated at 37°C. At appropriate time intervals, 200 µL samples were withdrawn and immediately added to 600 µL acetonitrile kept in an ice bath to precipitate the enzyme. The samples were vacuum dried (Speed Vac plus, Savant, NY) at low temperature, reconstituted with HPLC (high performance liquid chromatography) mobile phase solution (methanol to isopropanol ratio of 50:50), vortexed, and centrifuged at 3000 RPM for 10 minutes. The supernatant was analyzed with a reverse phase HPLC system based on an existing method.[9] A liposomal formulation of cholesterol oleate was prepared as a control and underwent the same process as described. The HPLC method specifically separated BCH and cholesterol oleate from their degradation product, cholesterol, and thus was suitable for the stability studies.[9]

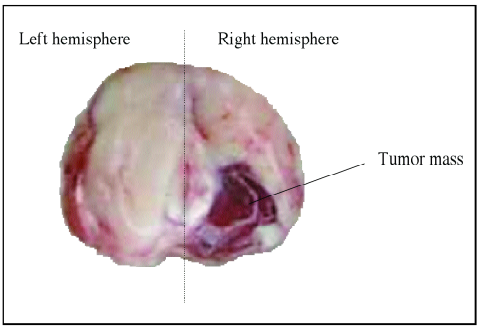

Figure 2. Picture of a rat brain with developed brain tumor after implantation of 9L glioma cells (16 days after implantation). The tumor tissue was located on the upper part of the right hemisphere.

RESULTS

The size of the BCH liposomes was measured by photon correlation spectroscopy (number weighted mean) and a bimodal distribution was observed. Approximately 91% of the vesicles had a mean diameter of 49.31 ± 6.30 nm and the remaining 9% of the vesicles had a mean diameter of 170.10 ± 21.32 nm. The mean BCH concentration in the liposomal formulation was about 150 µg/mL.

After 14 days post-implantation of tumor cells in the rat brains, the tumor was readily visualized (Figure 2). Histological examination of the brain tissue indicated the presence of large pleomorphic nuclei and abnormal mitotic figures (Figure 3). Physical changes were also observed in the tumor-bearing rats, including weight loss and abnormal yellowish nasal excretion, further confirming the presence of brain tumor. The concentration of BCH in tumor tissues after intracerebral administration at the tumor site was 27.70 ± 5.54, 12.51

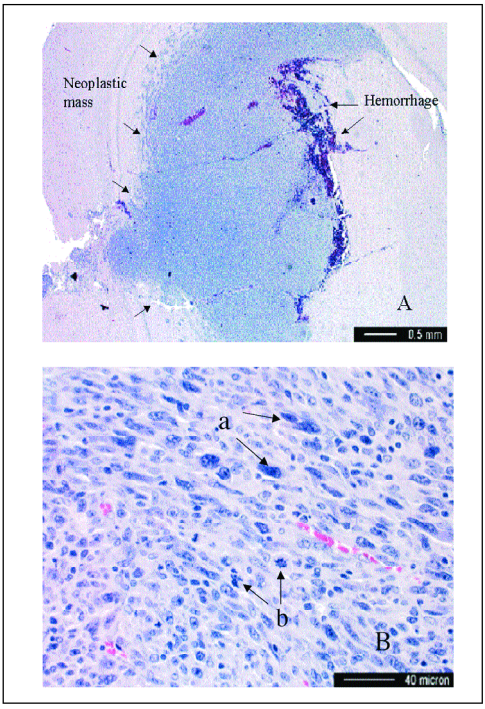

Figure 3. Histological examination indicated presence of tumor in a rat brain (hematoxylin-and-eosin staining): (A) brain section through cerebrum, neoplastic mass (arrows) with hemorrhage of one margin (arrowheads); (B) neoplastic mass, indicated by the large pleomorphic nuclei (a) and abnormal mitotic figures (b).

± 2.51, 9.13 ± 1.83, and 5.47 ± 1.15 µg/gm tissue at 2 6, 8, and 14 hours, respectively. The concentration of BCH in the surrounding normal tissues was 5.07 ± 1.01, 7.99 ± 1.59, 8.15 ± 1.71, and 5.42 ± 1.19 µg/gm tissue at 2, 6, 8, and 14 hours, respectively (Figure 4). Ratios of BCH concentrations in tumor tissue and in surrounding normal tissue were 5.46, 1.56, 1.12, and 1.01 at 2, 6, 8, and 14 hours, respectively (Figure 5). In the in vivo affinity study when the tumor tissue and normal tissue were sampled at 2 mm from the intracerebral BCH injection site in opposite directions, the concentration of BCH in tumor tissue and in normal tissue were 4.45 ± 0.89 µg/gm tissue and 3.91 ± 0.78 µg/gm tissue, respectively (Figure 6).

The chemical stability of BCH in the presence of cholesterol esterase and lipoprotein lipase was examined and the results are presented in Figure 7. BCH was formulated in liposomes to increase its aqueous solubility and incubated with cholesterol esterase and lipoprotein lipase, while cholesterol oleate liposomal formulation was used as the control. After 24 hours, 90.10% and 99.51% of BCH remained intact in CE and LPL media, respectively. On the other hand, only 78.01% and 47.26% of cholesterol oleate remained intact in CE and LPL media, respectively.

DISCUSSION

Recently, BCH was synthesized in our laboratory as a new anticancer conjugate for boron neutron capture therapy. BCH was designed based on the fact that rapidly dividing tumor cells require more cholesterol to construct cell membranes. It is known that many types of tumor cells possess elevated LDL receptor activities in order to acquire more cholesterol carried by low-density lipoprotein (LDL).[10-13] A recent study at UCSF reported that seven human glioblastoma multiforme cell lines had very high numbers of low-density lipoprotein (LDL) receptors per cell, indicating that the LDL pathway may provide a method

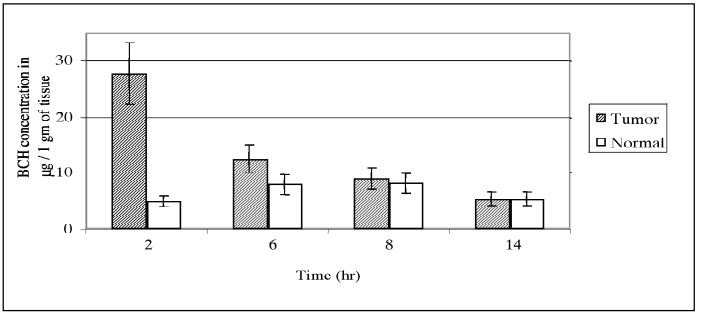

Figure 4. BCH concentrations in the brain at 2, 6, 8, and 14 hours after it was intracerebrally injected at the tumor site.

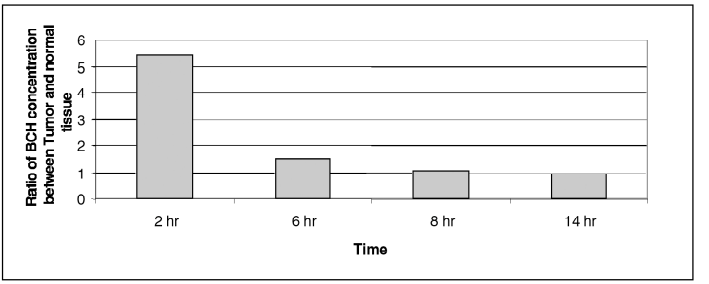

Figure 5. Ratios of BCH concentrations between tumor tissue and normal tissue at 2, 6, 8 and 14 hours after the intracerebral administration.

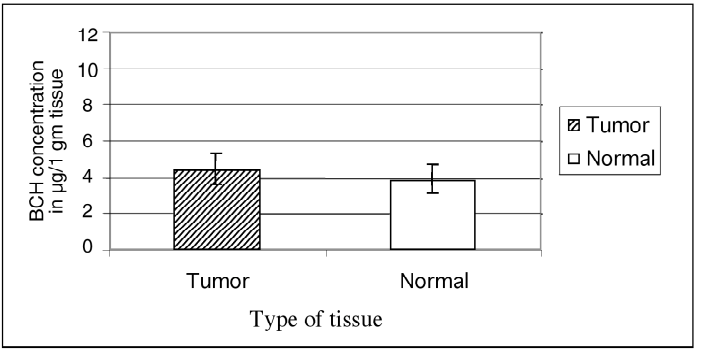

Figure 6. BCH concentrations in tumor tissue and normal tissue sampled at the opposite direction of the BCH injection site, respectively.

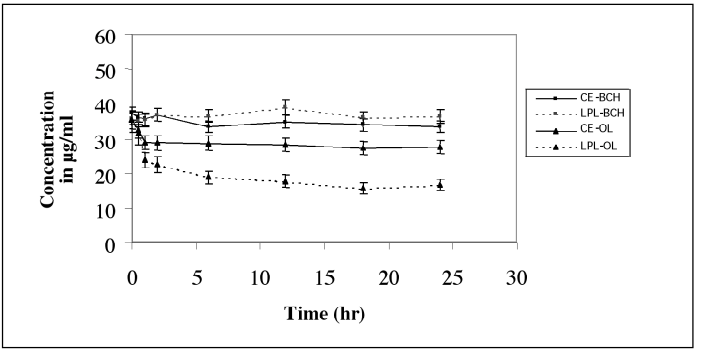

Figure 7. Degradation profiles of BCH and cholesterol oleate (as the control) in cholesterol esterase and lipoprotein lipase.

for selectively targeting anticancer compounds to glioblastoma multiforme cells.[14] Correspondingly, the design and synthesis of anticancer compounds in our laboratory have focused on derivatives of cholesteryl esters including BCH. In our cell culture studies, the amount of BCH taken by the glioma cells (SF-763 and SF-767) was much more (up to 14 times) than that by the normal brain neuron cells. The cellular uptake of BCH by the glioma cells after 16 hours of incubation was 283.3 ± 38.9 µg boron/g cells and 264.0 ± 36.5 µg boron/g cells, respectively, about 10 times higher than the required boron level for effective boron neutron capture therapy.[5] A mechanistic study indicated an inhibition of BCH uptake in the presence of the anti-LDL receptor antibody and a positive correlation of BCH uptake with the amount of LDL in the culture medium, strongly suggesting the involvement of LDL receptor during the uptake.[15]

Following positive results from the cell culture studies, the present study focused on the in vivo investigation of BCH for its diffusion in rat brain after intracerebral administration using a tumor-bearing rat model. Many previous studies have indicated that the intracerebral delivery of anticancer compounds can significantly enhance their efficacy.[8,16-18] In this paper, tumor-bearing rats obtained by implanting 9L brain glioma cells were used to evaluate the distribution of BCH at different time points and to examine the in vivo BCH affinity to tumor cells and normal cells. BCH concentration in tumor tissue (site of injection) decreased from 27.70 ± 5.54 µg/gm tissue to 12.51 ±

2.51 μg/gm tissue at 2 hours and 6 hours, respectively. The decline of BCH concentration, greater than 54% within 4 hours, indicates that BCH diffused from the site of injection rapidly. In addition, the BCH concentration in neighboring normal tissues increased from 5.07 ± 1.01 μg/gm tissue to 7.99 ± 1.59 μg/gm tissue at 2 hours and 6 hours, respectively, due to the diffusion of BCH from the injection site to the neighboring normal tissues. The result is in agreement with the brain distribution pattern of boroncaptate in tumor bearing rats after intracerebral injection.[8] Based on the diffusion study, the results also provided suggestions for a suitable irradiation time window to allow BCH to diffuse to most of tumor cells but keep the ratio of BCH concentrations in tumor tissue and normal tissue sufficiently high. Due to the rapid BCH diffusion in brain, an adequate radiation window appeared to be less than 2 hours. In our study regarding the in vivo affinity of BCH to tumor and normal tissues, the BCH concentration in tumor tissues and normal tissues, both sampled 2 mm away from the BCH injection site, was 4.45 ± 0.89 μg/gm tissue and 3.91 ± 0.78 μg/gm tissue, correspondingly. The small difference in BCH concentrations, however, did not yield a statistical significance at 95% confidence level (student's *t* test), which again appeared to contribute to the rapid diffusion of BCH in brain tissues.

Because BCH is composed of a cholesterol unit linked to the anticancer carborane moiety via an ester bond, its stability in the presence of hydrolytic enzymes, such as CE and LPL, in the brain may be questionable[19,20-22] and needs to be examined in parallel with the diffusion study. The suitability of using phospholipid vesicles for the hydrolytic study of cholesteryl ester has been demonstrated.[23,24] Our results indicated that BCH in liposomal formulation was relatively stable after incubated with CE and LPL, with 90.10% and 99.51% of BCH remaining intact, respectively. In contrast, during the control study, cholesterol oleate in liposomal formulation showed only 78.01% and 47.26% remaining intact for CE and LPL, respectively, under the same conditions (Figure 7). The presence of a bulky group near to the ester bond is known to provide protection from hydrolysis by steric hindrance.[25,26] Thus, BCH stability in the presence of CE and LPL could be attributed to the existence of the bulky carborane group next to the ester bond. Absence of a bulky group in cholesterol oleate apparently led to its instability. The hydrolytic effect of CE and LPL, however, was significantly different on BCH and cholesterol oleate. The stability difference between BCH and cholesterol oleate in the presence of CE was 12.09%. The stability difference in the presence of LPL, however, was much greater (52.25%).

In conclusion, the new cholesteryl ester anticancer conjugate, BCH, was investigated for its in vivo diffusion and affinity in brain using a tumor-bearing rat model. The chemical stability of BCH in brain hydrolytic enzymes, CE and LPL, was also examined. The results indicated that BCH formulated in liposomes distributed from the site of intracerebral injection rapidly. The adequate radiation time window appeared to be less than 2 hours. A small but statistically insignificant difference was found between the BCH concentrations in tumor tissue and normal tissue, both sampled equidistant to the BCH injection site. BCH was stable in presence of cholesterol esterase and lipoprotein lipase, when compared with cholesterol oleate as the control.

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REFERENCES

1. Anderson RN. *Deaths: leading causes for 2000. Natl Vital Stat Rep*. 2002;50:1-85.
2. Zee-Cheng RK, Cheng CC. Delivery of anticancer drugs. *Methods Find Exp Clin Pharmacol* 1989;11:439-529.
3. Lu DR, Mehta SC, Chen W. Selective boron drug delivery to brain tumors for boron neutron capture therapy. *Adv Drug Deliv Rev*. 1997;26:231-247.
4. Peacock G, Ji B, Wang CK, Lu DR. Cell cultural studies for a carborane cholesteryl ester with conventional and PEG liposomes. *Drug Delivery.* 2003;10:29-34.
5. Peacock G, Sidwell R, Pan G, Oie S, Lu DR. In vitro uptake of a new cholesteryl carborane ester compound in human glioma cell lines. *J Pharm Sci*. 2004;93:13-19.
6. Ji B, Peacock G, Lu DR. Synthesis of cholesterol-carborane conjugate for targeted drug delivery. *Bioorganic Med Chem Lett.* 2002;12:2455-2458.
7. Alanazi F, Li H, Halpern DS, Øie S, Lu DR. Synthesis, preformulation and liposomal formulation of cholesteryl carborane esters with various fatty chains. *Int J Pharm.* 2003;225:189-197.
8. Ji B, Chen W, Lu DR, Halpern DS. Cell culture and animal studies for intracerebral delivery of borocaptate in liposomal formulation. *Drug Deliv.* 2001;8:13-17.
9. Sidwell R. Extraction and analysis by HPLC of the novel compound BCH in formulations, cell culture, and animal tissues [thesis]. Athens, Ga: University of Georgia; 2002.
10. Callahan DE, Forte TM, Afzal SM, et al. Boronated protoporphyrin (BOPP): localization in lysosomes of the human glioma cell line SF-767 with uptake modulated by lipoprotein levels. *Int J Radiat Oncol Biol Phys*. 1999;45:761-771.
11. Murakami M, Ushio Y, Mihara Y et al. Cholesterol uptake by human glioma-cell via receptor-mediated endocytosis of Low-density lipoprotein. *J Neurosurg.* 1990;73:760-767.
12. Rudling MJ, Angelin B, Peterson CO, Collins VP. Low-density lipoprotein receptor activity in human intracranial tumors and its relation to the cholesterol requirement. *Cancer Res.* 1990;50:483-487.
13. Lombardi P, Norata G, Maggi FM et al. Assimilation of LDL by experimental-tumors in mice. *Biochim Biophys Acta.* 1989;1003:301-306.
14. Maletinska L, Blakely EA, Bjornstad KA, et al. Human glioblastoma cell lines: Levels of low-density lipoprotein receptor and low-density lipoprotein receptor-related protein. *Cancer Res.* 2000;60:2300-2303.
15. Pan G. In vitro gene and drug delivery and targeting to human glioma cells by lipoprotein mimics [thesis]. Athens, Ga: University of Georgia; 2003.
16. McKeran RO, Firth G, Oliver S, Uttley D, O'Laoire S. A potential application for the intracerebral injection of drugs entrapped within liposomes in the treatment of human cerebral gliomas. *J Neurol Neurosurg Psychiatry*. 1985;48:1213-1219.
17. Firth G, Oliver AS, McKeran RO. Studies on the intracerebral injection of bleomycin free and entrapped within liposomes in the rat. *J Neurol Neurosurg Psychiatry.* 1984;47:585-589.
18. Oliver AS, Firth G, McKeran RO. Studies on the intracerebral injection of vincristine free and entrapped within liposomes in the rat. *J Neurol Sci*. 1985;68:25-30.
19. Johnson RC, Shah SM. Cholesterol ester metabolizing enzymes in human brain: properties, subcellular distribution and relative levels in various diseases conditions.
20. Nunez M, Peinado-Onsurbe J, Vilaro S, Llobera M. Lipoprotein lipase activity in Developing Rat brain areas. *Biol Neonate.* 1995;68:119-127.
21. Ogino T, Suzuki K. Specificities of human and rat brain enzymes of cholesterol ester metabolism toward very long chain acids: implication for biochemical pathogenesis of Adrenoleukodystrophy. *J Neurochem.* 1981;36:776-779.
22. Roussea A, Dubois G, Gatt S. Subcelluar distribution of diacylglycerol lipase in rat and mouse brain. *Neurochem Res*. 1983;8:417-422.
23. Brecher P, Chobanian J, Small DM, Chobanian AV. The use of phospholipid vesicles for in vitro studies on cholesteryl ester hydrolysis. *J Lipid Res.* 1976;17:239-247.
24. Ishii I, Onozaki R, Takahashi E, et al. Regulation of neutral cholesterol esterase activity by phospholipids containing negative charges in substrate liposome. *J Lipid Res*. 1995;36:2303-2310.
25. VanDort M, Schwendner SW, Skinner RW, Gross MD, Counsell RE. Potential tumor or organ-imaging agents. 24. Radioidinated pregenolone esters. *Steroid*. 1984;44:85-93.
26. Morrison RT, Body RN. *Organic chemistry*. 3rd ed. Boston, Mass: Allan and Bacon Inc; 1973:603.