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 (54) Title: SELECTIVE DENDRIMER DELIVERY TO BRAIN TUMORS

(57) **Abrégé/Abstract:**

A composition comprising poly(amidoamine) (PAMAM) hydroxyl- terminated dendrimers covalently linked to at least one therapeutic, prophylactic or diagnostic agent for the treatment or alleviation of one or more symptoms of a brain tumor have been developed. The dendrimers comprise one or more ethylene diamine-core poly(amidoamine) (PAMAM) hydroxyl-terminated generation-4, 5, 6, 7, 8, 9, or 10, most preferably generation 6 (G4-10-OH) dendrimers. The G6 dendrimers have demonstrated unexpectedly high uptake into the brain. The dendrimers provide a means for selective delivery through the blood brain barrier ("BBB") of chemotherapeutic, immunotherapeutic and palliative agents. The dendrimers also have the advantage that two different classes of compounds, having one or more mechanisms of action can be bound to the dendrimers, providing simultaneous delivery. The dendrimers may be administered alone by intravenous injection, or as part of a multi-prong therapy with radiation.

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(57) **Abstract:** A composition comprising poly(amidoamine) (PAMAM) hydroxyl-terminated dendrimers covalently linked to at least one therapeutic, prophylactic or diagnostic agent for the treatment or alleviation of one or more symptoms of a brain tumor have been developed. The dendrimers comprise one or more ethylene diamine-core poly(amidoamine) (PAMAM) hydroxyl-terminated generation-4, 5, 6, 7, 8, 9, or 10, most preferably generation 6 (G4-10-OH) dendrimers. The G6 dendrimers have demonstrated unexpectedly high uptake into the brain. The dendrimers provide a means for selective delivery through the blood brain barrier ("BBB") of chemotherapeutic, immunotherapeutic and palliative agents. The dendrimers also have the advantage that two different classes of compounds, having one or more mechanisms of action can be bound to the dendrimers, providing simultaneous delivery. The dendrimers may be administered alone by intravenous injection, or as part of a multi-prong therapy with radiation.

SELECTIVE DENDRIMER DELIVERY TO BRAIN TUMORS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Nos. 62/036,675 filed August 13, 2014, 62/036,839, filed August 13, 2014, and 62/059,240 filed October 3, 2015, incorporated hereby by reference.

FIELD OF THE INVENTION

[0002] The present invention is generally in the field of delivery of chemotherapeutic, immunotherapeutic and palliative drugs to the brain for treatment of brain tumors and associated symptoms.

BACKGROUND OF THE INVENTION

[0003] A brain tumor is an abnormal growth of tissue in the brain or central spine that can disrupt proper brain function. Doctors refer to a tumor based on where the tumor cells originated, and whether they are cancerous (malignant) or not (benign). The least aggressive type of brain tumor is often called a benign brain tumor. They originate from cells within or surrounding the brain, do not contain cancer cells, grow slowly, and typically have clear borders that do not spread into other tissue. Malignant brain tumors contain cancer cells and often do not have clear borders. They are considered to be life threatening because they grow rapidly and invade surrounding brain tissue. Tumors that start in cells of the brain are called primary brain tumors. Primary brain tumors may spread to other parts of the brain or to the spine, but rarely to other organs. Metastatic or secondary brain tumors begin in another part of the body and then spread to the brain. These tumors are more common than primary brain tumors and are named by the location in which they begin.

[0004] There are over 120 types of brain and central nervous system tumors. Brain and spinal cord tumors are different for everyone. They form in different areas, develop from different cell types, and may have different treatment options. Often, low-grade tumors (grade I and II), which are not aggressive, are treated with watchful monitoring or surgery alone. Though all

tumors are monitored with repeat scans, grade II tumors are watched more closely after surgery and over time to make sure there is no recurrence. Higher grade tumors (grade III and IV), which are malignant and can grow quickly, are more difficult to remove and require additional treatments beyond surgery, such as radiation or chemotherapy. Microscopic tumor cells can remain after surgery and will eventually grow back. All treatments, therefore, are intended to prolong and improve life for as long as possible.

[0005] For a low-grade brain tumor, surgery may be the only treatment needed especially if all of the tumor can be removed. If there is visible tumor remaining after surgery, radiation and chemotherapy may be used. For higher-grade tumors, treatment usually begins with surgery, followed by radiation therapy and chemotherapy. Additional treatment options for high-grade tumors include X-rays and other forms of radiation to destroy tumor cells or delay tumor growth; chemotherapy to kill rapidly dividing cells; targeted therapy which focuses on a specific element of a cell, such as molecules or pathways required for cell growth, in order to use them as a target; and locally or regionally delivered treatment that produces electric fields to disrupt the rapid cell division exhibited by cancer cells by creating alternating, “wave-like” electric fields that travel across their region of usage in different directions.

[0006] Successfully treating brain tumors can be challenging. The body’s blood-brain barrier normally protects the brain and spinal cord from harmful chemicals entering those structures through the bloodstream. However, this barrier also keeps out many types of chemotherapy. Surgery can be difficult if the tumor is near a delicate part of the brain or spinal cord. Even when the surgeon can completely remove the original tumor, there may be parts of the tumor remaining that are too small to be seen or removed during surgery. Radiation therapy can damage healthy tissue.

[0007] A brain tumor and its treatment often cause side effects. In addition to treatment to slow, stop, or eliminate the tumor, an important part of care is relieving a person’s symptoms and side effects. This approach is called palliative or supportive care, and it includes supporting the patient with his or her physical, emotional, and social needs. Pain medication to

help manage the pain from headaches, a common symptom of a brain tumor. Often, corticosteroids are used to lower swelling in the brain, which can lessen pain from the swelling without the need for prescription pain medications. Antiseizure medication is used to help control seizures.

[0008] Surgery is commonly used to remove all or part of brain tumors. Sometimes, surgery cannot be performed because the tumor is located in a place the surgeon cannot reach or is near a vital structure; these tumors are called inoperable.

[0009] The goal of chemotherapy can be to destroy cancer cells remaining after surgery, slow a tumor's growth, or reduce symptoms. A chemotherapy regimen usually consists of a specific number of cycles given over a set period of time. A patient may receive one drug at a time or combinations of different drugs at the same time. Common ways to give chemotherapy include a pill or capsule that is swallowed (orally) or by intravenous (IV). Some drugs are better at going through the blood-brain barrier, and these drugs are often used for a brain tumor because of this ability. Gliadel wafers are one way to give the drug carmustine, which involves placing the wafers in the area where the tumor was removed during surgery. For people with glioblastoma, the latest standard of care is radiation therapy with daily low-dose temozolomide (Temodar), followed by monthly doses of temozolomide after radiation therapy for six months to one year. A combination of three drugs, lomustine (CeeNU), procarbazine (Matulane), and vincristine (Vincasar) have been used along with radiation therapy. This approach has helped lengthen the lives of patients with grade III oligodendroglioma with a 1p19q co-deletion when given either before or right after radiation therapy. It has also been shown to lengthen lives of patients when given after radiation therapy for low-grade tumors that could not be completely removed with surgery. The side effects of chemotherapy depend on the individual and the dose used, but they can include fatigue, risk of infection, nausea and vomiting, hair loss, loss of appetite and diarrhea. These side effects usually go away once treatment is finished. Rarely, certain drugs may cause some hearing loss. Others may cause kidney damage.

Patients may be given extra fluid by IV to protect their kidneys. A complete list of cancer drugs can be found on the NCI website.

[0010] Anti-angiogenesis is focused on stopping angiogenesis, which is the process of making new blood vessels. Because a tumor needs the nutrients delivered by blood vessels to grow and spread, the goal of anti-angiogenesis therapies is to “starve” the tumor. Bevacizumab (Avastin) is an anti-angiogenesis therapy used to treat glioblastoma multiform when prior treatment has not worked.

[0011] A remission is when the tumor cannot be detected in the body. A remission can be temporary or permanent. For most primary brain tumors, despite imaging tests showing that the tumor growth is controlled or there are no visible signs of a tumor, it is common for a brain tumor to recur.

[0012] In glioma, tumor associated microglia/macrophages (TAM) have been shown to participate in tumor growth, tumor invasion, angiogenesis and immune system evasion. TAM is subjected to reprogramming in the tumor microenvironment, leading to an alternate immunosuppressive tumorigenic M2 phenotype. (da Fonseca AC, Badie B. Clin Dev Immunol 2013:264124). A variety of microglia/macrophage modulating molecules has been shown to switch the phenotype of TAMs and decrease glioma progression and increase survival in preclinical studies (El Andaloussi A, et, al. Glia 2006;54:526-35; Hussain SF, et, al. Cancer Res 2007;67:9630-6; Gabrusiewicz K, et, al. PLoS One 2011;6:e23902; Markovic DS, et, al. Brain Behav Immun 2011;25:624-8.). Target delivery of immunomodulatory molecules to TAMs may provide improved efficacy with reduced side effects.

[0013] Malignant glioma is the most common and most aggressive primary brain tumor and despite the advances in treatment, the median survival remains at 16.4 months. Key challenges faced in the development of effective therapies relate to (a) the ability of systemically delivered chemotherapeutic agents to penetrate the impaired blood brain tumor barrier (BBTB) and provide homogenous coverage across the entire solid tumor and (b) the ability to target specific cells. Although small molecule-based therapeutics can effectively distribute within the tumor tissue, they are limited by rapid tumor clearance and off-target extravasation potentially

leading to adverse effects. Recent advances in nanotechnology have provided selective tumor accumulation. However, the size of most nanoparticles limits extravasation and tumor penetration, thus limiting homogeneous solid tumor coverage. Careful tuning of particle size and surface charge has been attempted in order to enhance the nanoparticle distribution profile in subcutaneous tumors. Unfortunately, achieving homogeneous coverage of orthotopic brain tumors has proven even more challenging. This may be attributed to the lower permeability of the BBTB compared to the blood-tumor barrier (BTB) in a subcutaneous tumor, the heterogeneous intervascular spaces and the high interstitial pressure in brain tumors. Although, some strategies have attempted nanoparticle delivery through the BBTB via absorptive uptake; passive diffusion through the leaky BBTB fenestrations has only been demonstrated with molecules smaller than 20 nm and unhindered diffusion through the BBTB has been achieved with molecules of 7 nm, thus limiting systemic administration of most nanoparticle based therapeutics.

[0014] It is therefore an object of the present invention to provide an improved method and reagents for delivering drugs to treat brain tumors.

SUMMARY OF THE INVENTION

[0015] A composition comprising poly(amidoamine) (PAMAM) hydroxyl-terminated dendrimers covalently linked to or complexed with at least one therapeutic, prophylactic or diagnostic agent for the treatment or alleviation of one or more symptoms of a brain tumor have been developed. The composition contains one or more ethylene diamine-core poly(amidoamine) (PAMAM) hydroxyl-terminated generation-4, 5, 6, 7, 8, 9, or 10 (G4-10-OH) dendrimers. The G6 dendrimers have demonstrated unexpectedly high uptake, and uniform distribution in to the entire brain tumor. The dendrimers provide a means for selective delivery through the blood brain barrier ("BBB") of chemotherapeutic, immunotherapeutic and palliative agents. The dendrimers also have the advantage that multiple therapeutic, prophylactic, and/or diagnostic agents can be delivered with the same dendrimers. In one embodiment, the dendrimers are complexed with or conjugated to two different classes of compounds, providing simultaneous

delivery. The dendrimers may be administered alone by intravenous injection, or as part of a multi-prong therapy with radiation and/or surgery. In one embodiment, the dendrimers are covalently linked to at least one radiosensitizing agent, in an amount effective to suppress or inhibit the activity of DDX3 in the proliferative disease. In another embodiment, the dendrimers are covalently linked to at least one detectable moiety, in an amount effective to detect the tumor in the subject. In another embodiment, the dendrimer composition has multiple agents, such as a chemotherapeutic agent, immunotherapeutic agent, an anti-seizure agent, a steroid to decrease swelling, antibiotic, anti-angiogenic agent, and/or a diagnostic agent, complexed with or conjugated to the dendrimers.

[0016] The dendrimer composition is preferably administered systemically, most preferably via intravenous injection. The composition may be administered prior to or immediately after surgery, radiation, or both. The composition may be designed for treatment of specific types of tumors, such as gliomas, or through targeting tumors associated with microglia/macrophages (TAM).

[0017] The examples demonstrated that hydroxyl terminated PAMAM dendrimers demonstrate unique favorable pharmacokinetic characteristics in a glioblastoma tumor model following systemic administration. Dendrimers rapidly accumulate and are selectively retained in the tumor tissue. This is due at least in part to the small size and near neutral surface charge which allow homogeneous distribution of the dendrimer through the entire solid tumor. Dendrimers homogeneously distribute through the extracellular matrix reaching the entire tumor and peritumoral area. Dendrimers intrinsically target neuroinflammation and accumulate in the tumor associated microglia/macrophages (TAMs). Increasing the generation of dendrimers from 4 to 6 can significantly increase dendrimer accumulation in the tumor without affecting their homogeneous distribution and targeting of TAMs. The generation 4 and 6 hydroxyl terminated PAMAM dendrimers can leak through the blood brain tumor barrier and selectively accumulate in glioblastoma, not the peritumoral area, following systemic administration. However, the dendrimers also accumulate in the peritumoral area, thereby

having an effect on the migrating front of glioblastoma. These dendrimers intrinsically target tumor associated microglia/macrophages and are retained in these cells over at least 48 hours. There is no significant accumulation of dendrimers in the contralateral hemisphere ('healthy') where the dendrimers remain in the blood vessel lumen.

[0018] Generation 4 (G4) dendrimers rapidly and selectively accumulate and are retained in the tumor tissue despite their rapid clearance from the circulation. Based on fluorescence quantification and high resolution fluorescence microscopy dendrimers accumulate over the first 8 hours and are still retained in the tumor at 48 hours. Increasing the generation of dendrimers from 4 to 6 can significantly increase dendrimer accumulation, AUC and retention in the tumor ~100-fold without affecting their homogeneous distribution and targeting of TAMs.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Figures 1A-1C show a synthetic scheme for conjugating a small molecule, BLZ-945, to a G4 hydroxyl-terminated dendrimer.

[0020] Figures 2A and 2B are graphs illustrating D-Cy5 pharmacokinetics in the brain (tumor, peritumoral area and contralateral hemisphere) of a rodent 9L gliosarcoma model. Figure 1A is a graph of D-Cy5 concentration in brain areas 15 minutes, 1 hour, 4 hours, 8 hours, 24 hours and 48 hours following systemic administration. The accumulation is expressed as μg of D-Cy5 per g of tissue. Figure 1B is a graph of the area under the curve ("AUC") of D-Cy5. The AUC at 48 hours demonstrates the significant difference in dendrimer exposure between the tumor and the contralateral hemisphere.

[0021] Figure 3A is a graph of the linear curve fitting of brain quantification data. The R^2 for contralateral, peritumor and tumor are 0.98, 0.99 and 0.96 respectively. Figure 3B shows calculation of permeation constant (K_{in}) and initial volume of distribution (V_i) in tumor, peritumoral area and contralateral hemisphere based on brain pharmacokinetics data, Tissue (t)/serum (t) (mL/g) versus AUC serum t/Serum (t) (hr).

[0022] Figure 4A and 4B depict the characterization of microglia cells (population and activation) in a 9L gliosarcoma inoculated rodent brain using Imaris software. Figure 4A is a graph of the image based cell count of the Iba1+ microglia/macrophages population per mm² area in the tumor, ipsilateral hemisphere and contralateral hemisphere. Figure 4B is a graph of the image based measurement of microglia cell surface to volume ratio as an indication of activation and phagocytic activity of microglia/macrophages in healthy brain, contralateral hemisphere and ipsilateral hemisphere of a tumor inoculated brain and tumor tissue. Iba1+ cells D-Cy5 co-localization with TAMs and D-Cy5 co-localization with DAPI+ cells. Results are expressed as percent of the total DAPI+ cell population. There is no statistical significance between microglia uptake and cell uptake. Statistical * p<0.05; ** p<0.001 Statistical analysis is based on 3-5 different slices.

[0023] Figure 5 is a graph of the D-Cy5 cell localization analysis 24 hours following administration using image based measurement of Iba-1+ cells D-Cy5 co-localization with Iba1+ TAMs and D-Cy5 co-localization with DAPI+ cells. Results are expressed as percent of the total DAPI+ cell population. There is no statistical significance between microglia uptake and cell uptake.

[0024] Figures 6A-6C are graphs based on the fluorescence based quantification of D-Cy5 in major organs (brain, liver, lung, spleen, heart, and kidney), serum and urine of 9L gliosarcoma rodent model 24 hours following D-Cy5 administration. Figure 6A is the biodistribution is expressed in percent of injected dose per organ: D-Cy5 accumulation in kidney, urine and other organs. Figure 6B is a graph of the time dependent concentration of D-Cy5 in spleen, liver, kidney and serum. Concentration is expressed in percent of injected dose per g of tissue. Figure 6C is a graph of the fluorescence based quantification of the plasma pharmacokinetics of D-Cy5.

[0025] Figures 7A-7C are graphs of the comparison of accumulation (μg of dendrimer / g of brain tissue) (Figure 7A) between G6 and G4 dendrimers in a tumor bearing brain as a function of time. The accumulation of G4 dendrimers in the tumor/peritumor peaked around 8 hours post injection, and gradually decreased, while G6 dendrimer concentration in tumor/peritumor

continuously increased. At 48 hours, G6 concentration was almost 100 fold higher than G4 dendrimers concentration in the tumor; the area under curve (AUC) plot (Figure 7B) which demonstrates G6 dendrimers have around 100 fold higher brain tumor exposure than G4 dendrimers within 48 hours.

When the dendrimer concentration in the brain is normalized by the dendrimer concentration in the serum, the brain to serum ratio is an indication of the brain targeting ability. G6 dendrimers showed higher tumor targeting ability than G4 dendrimers at all the time points (Figure 7C).

[0026] Figures 8A and 8B are graphs showing the G4 and G6 dendrimer concentration in serum and major organs: kidney, liver, spleen as a function of time. Figure 8A shows that G6 dendrimers showed higher serum concentration and prolonged serum half-life than G4 dendrimers, which contributed to the higher tumor accumulation and targeting of G6 dendrimers. The dendrimer concentration was demonstrated as percentage of total injected dose per milliliter of serum. Figure 8B showed that for G4 dendrimers, kidney had most dendrimer accumulation (20%-30%), significantly higher than dendrimer accumulation in liver and spleen (~0.3%) at different time points. For G6 dendrimers, the increase of size greatly decreased the renal filtration and kidney accumulation. The kidney concentration of G6 dendrimers was more than 10 fold less than G4 dendrimers (~1%), and started to show clearance from kidney starting from 48 hours. The liver accumulation of G6 dendrimers was similar to G4 dendrimers, while spleen showed ~5 fold higher accumulation, possibly due to the increased uptake by monocytes.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

[0027] The term "therapeutic agent" refers to an agent that can be administered to prevent or treat one or more symptoms of a disease or disorder. Examples include, but are not limited to, a nucleic acid, a nucleic acid analog, a small molecule, a peptidomimetic, a protein, peptide, carbohydrate or sugar, lipid, or surfactant, or a combination thereof.

[0028] The term "treating" refers to preventing or alleviating one or more symptoms of a disease, disorder or condition. Treating the disease or

condition includes ameliorating at least one symptom of the particular disease or condition, even if the underlying pathophysiology is not affected, such as treating the pain of a subject by administration of an analgesic agent even though such agent does not treat the cause of the pain.

[0029] The phrase "pharmaceutically acceptable" refers to compositions, polymers and other materials and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. The phrase "pharmaceutically acceptable carrier" refers to pharmaceutically acceptable materials, compositions or vehicles, such as a liquid or solid filler, diluent, solvent or encapsulating material involved in carrying or transporting any subject composition, from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of a subject composition and not injurious to the patient.

[0030] The phrase "therapeutically effective amount" refers to an amount of the therapeutic agent that produces some desired effect at a reasonable benefit/risk ratio applicable to any medical treatment. The effective amount may vary depending on such factors as the disease or condition being treated, the particular targeted constructs being administered, the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art may empirically determine the effective amount of a particular compound without necessitating undue experimentation.

II. Formulation

A. Dendrimers

[0031] The term "dendrimer" as used herein includes, but is not limited to, a molecular architecture with an interior core, interior layers (or "generations") of repeating units regularly attached to this initiator core, and an exterior surface of terminal groups attached to the outermost generation. Examples of dendrimers include, but are not limited to, PAMAM, polyester, polylysine, and PPI. The PAMAM dendrimers can have carboxylic, amine and hydroxyl terminations and can be any generation of dendrimers

including, but not limited to, generation 1 PAMAM dendrimers, generation 2 PAMAM dendrimers, generation 3 PAMAM dendrimers, generation 4 PAMAM dendrimers, generation 5 PAMAM dendrimers, generation 6 PAMAM dendrimers, generation 7 PAMAM dendrimers, generation 8 PAMAM dendrimers, generation 9 PAMAM dendrimers, or generation 10 PAMAM dendrimers. Dendrimers suitable for use include, but are not limited to, polyamidoamine (PAMAM), polypropylamine (POPAM), polyethylenimine, polylysine, polyester, iptycene, aliphatic poly(ether), and/or aromatic polyether dendrimers. Each dendrimer of the dendrimer complex may be of similar or different chemical nature than the other dendrimers (e.g., the first dendrimer may include a PAMAM dendrimer, while the second dendrimer may comprise a POPAM dendrimer). In some embodiments, the first or second dendrimer may further include an additional agent. The multiarm PEG polymer includes a polyethylene glycol having at least two branches bearing sulfhydryl or thiopyridine terminal groups; however, embodiments disclosed herein are not limited to this class and PEG polymers bearing other terminal groups such as succinimidyl or maleimide terminations can be used. The PEG polymers in the molecular weight 10 kDa to 80 kDa can be used.

[0032] A dendrimer complex includes multiple dendrimers. For example, the dendrimer complex can include a third dendrimer; wherein the third-dendrimer is complexed with at least one other dendrimer. Further, a third agent can be complexed with the third dendrimer. In another embodiment, the first and second dendrimers are each complexed to a third dendrimer, wherein the first and second dendrimers are PAMAM dendrimers and the third dendrimer is a POPAM dendrimer. Additional dendrimers can be incorporated without departing from the spirit of the invention. When multiple dendrimers are utilized, multiple agents can also be incorporated. is not limited by the number of dendrimers complexed to one another.

[0033] As used herein, the term "PAMAM dendrimer" means poly(amidoamine) dendrimer, which may contain different cores, with amidoamine building blocks. The method for making them is known to those of skill in the art and generally, involves a two-step iterative reaction

sequence that produces concentric shells (generations) of dendritic β -alanine units around a central initiator core. This PAMAM core-shell architecture grows linearly in diameter as a function of added shells (generations). Meanwhile, the surface groups amplify exponentially at each generation according to dendritic-branching mathematics. They are available in generations G0 - 10 with 5 different core types and 10 functional surface groups. The dendrimer-branched polymer may consist of polyamidoamine (PAMAM), polyester, polyether, polylysine, or polyethylene glycol (PEG), polypeptide dendrimers.

[0034] In accordance with some embodiments, the PAMAM dendrimers used can be generation 4 dendrimers, or more, with hydroxyl groups attached to their functional surface groups. The multiarm PEG polymer comprises polyethylene glycol having 2 and more branches bearing sulfhydryl or thiopyridine terminal groups; however, embodiments are not limited to this class and PEG polymers bearing other terminal groups such as succinimidyl or maleimide terminations can be used. The PEG polymers in the molecular weight 10 kDa to 80 kDa can be used.

[0035] In some embodiments, the dendrimers are in nanoparticle form and are described in detail in international patent publication No. WO2009/046446.

Preparation of PAMAM-BLZ-945

[0036] As a non-limiting example, below is a synthetic scheme for conjugating a small molecule, BLZ-945, to a hydroxyl-terminated fourth generation PAMAM dendrimer (PAMAM-OH), using acetic acid, 2-(2-cyclooctyn-1-yloxy) acid and 2-azidoacetic acid as linkers. See Figures 1A-1C

[0037] Initially, the hydroxyl-terminated fourth generation PAMAM dendrimer (PAMAM-OH) is functionalized into clickable bifunctional dendrimer (intermediate 1) with 9 clickable groups on the surface using 2-(2-cyclooctyn-1-yloxy) acid (Figure 1A). BLZ-945 is reacted with 2-azidoacetic acid to form azide-functionalized intermediate via an ester bond (Figure 1B). The resulting azide on the 2-azidoacetyl linker is further reacted with the clickable groups of the bifunctional dendrimer to get dendrimer-BLZ-945

conjugate. (Figure 1C) There are approximately nine molecules of BLZ-945 conjugated to one molecule of the dendrimer.

[0038] The scheme described above is not limited to BLZ-945. Other small molecules, for example, a small molecule inhibitor of a Signal Transducer and Activator of Transcription (STAT) protein such as WP1066, and other small molecules such as minocycline and cyclosporine A can be conjugated to the dendrimers as immunomodulatory molecules for TAMs targeting therapies.

B. Coupling Agents and Spacers

[0039] Dendrimer complexes can be formed of therapeutically active agents or compounds (hereinafter "agent") conjugated or attached to a dendrimer or multiarm PEG. The attachment can occur via an appropriate spacer that provides a disulfide bridge between the agent and the dendrimer. The dendrimer complexes are capable of rapid release of the agent in vivo by thiol exchange reactions, under the reduced conditions found in body.

[0040] The term "spacers" as used herein is intended to include compositions used for linking a therapeutically active agent to the dendrimer. The spacer can be either a single chemical entity or two or more chemical entities linked together to bridge the polymer and the therapeutic agent or imaging agent. The spacers can include any small chemical entity, peptide or polymers having sulfhydryl, thiopyridine, succinimidyl, maleimide, vinylsulfone, and carbonate terminations.

[0041] The spacer can be chosen from among a class of compounds terminating in sulfhydryl, thiopyridine, succinimidyl, maleimide, vinylsulfone and carbonate group. The spacer can comprise thiopyridine terminated compounds such as dithiodipyridine, N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP), Succinimidyl 6-(3-[2-pyridyldithio]-propionamido)hexanoate LC-SPDP or Sulfo-LC-SPDP. The spacer can also include peptides wherein the peptides are linear or cyclic essentially having sulfhydryl groups such as glutathione, homocysteine, cysteine and its derivatives, arg-gly-asp-cys (RGDC) (SEQ ID NO:1), cyclo(Arg-Gly-Asp-d-Phe-Cys) (c(RGDfC)) (SEQ ID NO:2), cyclo(Arg-Gly-Asp-D-Tyr-Cys)

(SEQ ID NO:3), cyclo(Arg-Ala-Asp-d-Tyr-Cys) (SEQ ID NO:4). The spacer can be a mercapto acid derivative such as 3 mercapto

propionic acid, mercapto acetic acid, 4 mercapto butyric acid, thiolan-2-one, 6 mercaptohexanoic acid, 5 mercapto valeric acid and other mercapto derivatives such as 2 mercaptoethanol and 2 mercaptoethylamine. The spacer can be thiosalicylic acid and its derivatives, (4-succinimidyl-oxycarbonyl-methyl-alpha-2-pyridylthio)toluene, (3-[2-pyridithio]propionyl hydrazide, The spacer can have maleimide terminations wherein the spacer comprises polymer or small chemical entity such as bis-maleimido diethylene glycol and bis-maleimido triethylene glycol, Bis-Maleimidoethane, bismaleimidohexane. The spacer can comprise vinylsulfone such as 1,6-Hexane-bis-vinylsulfone. The spacer can comprise thioglycosides such as thioglucose. The spacer can be reduced proteins such as bovine serum albumin and human serum albumin, any thiol terminated compound capable of forming disulfide bonds The spacer can include polyethylene glycol having maleimide, succinimidyl and thiol terminations.

[0042] The therapeutically active agent, imaging agent, and/or targeting moiety can be either covalently attached or intra-molecularly dispersed or encapsulated. The dendrimer is preferably a PAMAM dendrimer up to generation 10, having carboxylic, hydroxyl, or amine terminations. The PEG polymer is a star shaped polymer having 2 or more arms and a molecular weight of 10 kDa to 80 kDa. The PEG polymer has sulfhydryl, thiopyridine, succinimidyl, or maleimide terminations. The dendrimer is linked to the targeting moiety, imaging agents, and/or therapeutic agents via a spacer ending in disulfide, ester or amide bonds.

C. Therapeutic, Prophylactic and Diagnostic Agents

[0043] The term "dendrimer complexes" as used herein refers to the dendrimer conjugated to or complexed with one or more therapeutic, prophylactic, or diagnostic agent. The dendrimer complex, when administered by i.v. injection, can preferentially cross the blood brain barrier (BBB) only under diseased condition and not under normal conditions. Preferably the agent(s) is attached or conjugated to PAMAM dendrimers or multiarm PEG, which are capable of preferentially releasing the drug intracellularly under the reduced conditions found *in vivo*. The dendrimer complexes linked to an agent can be used to perform several

functions including targeting, localization at a diseased site, releasing the drug, and imaging purposes. The dendrimer complexes can be tagged with or without targeting moieties such that a disulfide bond between the dendrimer and the agent or imaging agent is formed via a spacer or linker molecule.

[0044] Representative therapeutic (including prodrugs), prophylactic or diagnostic agents can be peptides, proteins, carbohydrates, nucleotides or oligonucleotides, small molecules, or combinations thereof. Representative oligonucleotides include siRNAs, microRNAs, DNA, and RNA.

[0045] The term “chemotherapeutic agent” generally includes pharmaceutically or therapeutically active compounds that work by interfering with DNA synthesis or function in cancer cells. Based on their chemical action at a cellular level, chemotherapeutic agents can be classified as cell-cycle specific agents (effective during certain phases of cell cycle) and cell-cycle nonspecific agents (effective during all phases of cell cycle). Examples of chemotherapeutic agents include alkylating agents, angiogenesis inhibitors, modulators of tumor immune response, aromatase inhibitors, antimetabolites, anthracyclines, antitumor antibiotics, platinum compounds, topoisomerase inhibitors, radioactive isotopes, radiosensitizing agents, checkpoint inhibitors, PD1 inhibitors, APRKinase inhibitors, plant alkaloids, glycolytic inhibitors and prodrugs thereof.

[0046] Representative chemotherapeutics commonly used in treating brain tumors include taxols such as paclitaxel, BCNU, camptothecin, doxycycline, cisplatin, and derivatives, analogues and prodrugs thereof.

[0047] Examples of PD-1 inhibitors include, for example, MDX-1106 is a genetically engineered, fully human immunoglobulin G4 (IgG4) monoclonal antibody specific for human PD-1, and pembrolizumab, recently approved by the US FDA.

[0048] Therapeutic agents can include agents which enhance the effect of a different therapy, such as radiation. As used herein, the term “a radiation dose sensitizer” means any agent, which when contacted with a cell, population of cell or tissue, increases the susceptibility of that cell, population of cell or tissue to ionizing radiation. In some embodiments, the

radiosensitizer is a DDX3 inhibitor, such as the compound RK-33, or a salt, solvate, stereoisomer, or derivative thereof.

[0049] Therapeutic agents include agents which alleviate one or more symptoms of the brain tumor. For example, agents which reduce swelling associated with the tumor may be delivered via the dendrimers. Examples include anti-inflammatory agents such as steroids, for example, methyl prednisone, dexamethasone, and fluocinolone acetonide, non-steroidal anti-inflammatory agents such as COX-2 inhibitors, gold compound anti-inflammatory agents, immunosuppressive agents, salicylate anti-inflammatory agents, ranibizumab, minocycline, and rapamycin. Other anti-inflammatory drugs include nonsteroidal drug such as indomethacin, aspirin, acetaminophen, diclofenac sodium and ibuprofen.

[0050] A peptide drug can be any sequence that is active on TAMs or cancer cells. Examples include Peptides (M2pep with the sequence YEQDPWGVKWWY (SEQ ID NO:5) and scM2pep with the sequence WEDYQWPVYKGW (SEQ ID NO:6)) with a Lys3Gly3Ser linker and a C-terminal biotin tag were purchased from Elim Biopharmaceuticals at >95% purity. KLA materials were synthesized and purified at >95% purity as follows: M2pepKLA (YEQDPWGVKWWYGGGS-D[KLAKLAK]₂ (SEQ ID NO:7)), scM2pepKLA (WEDYQWPVYKGWSGGGS-D[KLAKLAK]₂ (SEQ ID NO:8)), and KLA (D[KLAKLAK]₂ (SEQ ID NO:9)).

[0051] Examples for immunotherapeutic agents targeting TAMs can include colony stimulating factor-1 (CSF-1) receptor inhibitor such as BLZ-945 and PLX3397, MAPKinase inhibitors such as PD98059, a small molecule inhibitor of STAT (*e.g.* WP1066), Minocycline, and cyclosporine A.

[0052] Other exemplary therapeutic agents include vasodilators and anti-infective agents. Antibiotics include beta-lactams such as penicillin and ampicillin, cephalosporins such as cefuroxime, cefaclor, cephalexin, cephhydroxil, cefpodoxime and proxetil, tetracycline antibiotics such as doxycycline and minocycline, macrolide antibiotics such as azithromycin,

erythromycin, rapamycin and clarithromycin, fluoroquinolones such as ciprofloxacin, enrofloxacin, ofloxacin, gatifloxacin, levofloxacin and

norfloxacin, tobramycin, colistin, or aztreonam as well as antibiotics which are known to possess anti-inflammatory activity, such as erythromycin, azithromycin, or clarithromycin. Other agents having activity as anti-excitotoxic agents such as valproic acid, D-aminophosphonovalerate, D-aminophosphonoheptanoate, inhibitors of glutamate formation/release, such as baclofen, and NMDA receptor antagonists can also be administered.

[0053] In some embodiments, the molecules can include antibodies, for example, daclizumab, bevacizumab (AVASTIN®), ranibizumab (LUCENTIS®), basiliximab, ranibizumab, and pegaptanib sodium or peptides like SN50, and antagonists of NF.

[0054] Exemplary diagnostic agents include paramagnetic molecules, fluorescent compounds, magnetic molecules, and radionuclides, x-ray imaging agents, and contrast media. These may also be ligands or antibodies which are labelled with the foregoing or bind to labelled ligands or antibodies which are detectable by methods known to those skilled in the art.

[0055] Exemplary diagnostic agents include dyes, fluorescent dyes, Near infra-red dyes, SPECT imaging agents, PET imaging agents and radioisotopes. Representative dyes include carbocyanine, indocarbocyanine, oxacarbocyanine, thüicarbocyanine and merocyanine, polymethine, coumarine, rhodamine, xanthene, fluorescein, boron~dipyrromethane (BODIPY), Cy5, Cy5.5, Cy7, VivoTag-680, VivoTag-S680, VivoTag-S750, AlexaFluor660, AlexaFluor680, AlexaFluor700, AlexaFluor750, AlexaFluor790, Dy677, Dy676, Dy682, Dy752, Dy780, DyLight547, Dylight647, HiLyte Fluor 647, HiLyte Fluor 680, HiLyte Fluor 750, IRDye 800CW, IRDye 800RS, IRDye 700DX, ADS780WS, ADS830WS, and ADS832WS.

[0056] Representative SPECT or PET imaging agents include chelators such as di-ethylene tri-amine penta-acetic acid (DTPA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), di-amine dithiols, activated mercaptoacetyl-glycyl-glycyl-glycine (MAG3), and hydrazidonicotinamide (HYNIC).

[0057] Representative isotopes include Tc-94m, Tc-99m, In-111, Ga-67, Ga-68, Gd³⁺, Y-86, Y-90, Lu-177, Re-186, Re-188, Cu-64, Cu-67, Co-55,

Co-57, F-18, Sc-47, Ac-225, Bi-213, Bi-212, Pb-212, Sm-153, Ho-166, and Dy-166.

[0058] Targeting moieties include folic acid, RGD peptides either linear or cyclic, TAT peptides, LHRH and BH3.

D. Devices and Formulations

[0059] The dendrimers can be administered parenterally by subdural, intravenous, intra-amniotic, intraperitoneal, or subcutaneous routes.

[0060] The carriers or diluents used herein may be solid carriers or diluents for solid formulations, liquid carriers or diluents for liquid formulations, or mixtures thereof.

[0061] For liquid formulations, pharmaceutically acceptable carriers may be, for example, aqueous or non-aqueous solutions, suspensions, emulsions or oils. Parenteral vehicles (for subcutaneous, intravenous, intraarterial, or intratissue injection) include, for example, sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, and injectable organic esters such as ethyl oleate. Aqueous carriers include, for example, water, alcoholic/aqueous solutions, cyclodextrins, emulsions or suspensions, including saline and buffered media.

[0062] The dendrimers can also be administered in an emulsion, for example, water in oil. Examples of oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, mineral oil, olive oil, sunflower oil, fish-liver oil, sesame oil, cottonseed oil, corn oil, olive, petrolatum, and mineral. Suitable fatty acids for use in parenteral formulations include, for example, oleic acid, stearic acid, and isostearic acid. Ethyl oleate and isopropyl myristate are examples of suitable fatty acid esters.

[0063] Formulations suitable for parenteral administration can include antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Intravenous vehicles can include fluid and nutrient replenishers, electrolyte replenishers

such as those based on Ringer's dextrose. In general, water, saline, aqueous dextrose and related sugar solutions, and glycols such as propylene glycols or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions.

[0064] Injectable pharmaceutical carriers for injectable compositions are well-known to those of ordinary skill in the art (see, e.g., *Pharmaceutics and Pharmacy Practice*, J.B. Lippincott Company, Philadelphia, PA, Banker and Chalmers, eds., pages 238-250 (1982), and *ASHP Handbook on Injectable Drugs*, Trissel, 15th ed., pages 622-630 (2009)).

[0065] Formulations for convection enhanced delivery (“CED”) include solutions of low molecular weight salts and sugars such as mannitol.

III. Methods of Treatment

A. Disorders or Diseases to be Treated

[0066] The dendrimer complex composition, including dendrimers linked to one or more therapeutic, prophylactic and/or diagnostic agents, can selectively target microglia and astrocytes. Effective blood-brain tumor barrier (BBTB) penetration and uniform solid tumor distribution significantly enhance therapeutic delivery to brain tumors. Hydroxyl-functionalized, generation-4 or 6 poly(amidoamine) (PAMAM) dendrimers, with their small size, near neutral surface charge, selectively localize in cells associated with neuroinflammation.

[0067] As used herein, the term “proliferative disease” includes cancer and other diseases such as benign and malignant neoplasias and hyperplasias. The term cancer, includes cancers of the CNS and brain, including, but not limited to, gliomas, glioblastoma, gliosarcoma, astrocytoma, oligodendroglioma, ependymoma, meningioma, medulloblastoma, ganglioma, Schwannoma, craniopharyngioma, cordomas and pituitary tumors.

[0068] The tumors may also be of a different origin than the brain. For example, the tumors may have originated as alveolar rhabdomyosarcoma, bone cancer, breast cancer, cancer of the anus, anal canal, or anorectum, cancer of the eye, cancer of the intrahepatic bile duct, cancer of the joints, cancer of the neck, gallbladder, or pleura, cancer of the nose, nasal cavity, or

middle ear, cancer of the oral cavity, cancer of the vulva, colon cancer, esophageal cancer, cervical cancer, gastrointestinal carcinoid tumor.

Hodgkin lymphoma, hypopharynx cancer, kidney cancer, larynx cancer, liver cancer, lung cancer, malignant mesothelioma, melanoma, multiple myeloma, nasopharynx cancer, non-Hodgkin lymphoma, ovarian cancer, pancreatic cancer, peritoneum, omentum, and mesentery cancer, pharynx cancer, prostate cancer, rectal cancer, renal cancer (e.g., renal cell carcinoma (RCC)), small intestine cancer, soft tissue cancer, stomach cancer, testicular cancer, thyroid cancer, ureter cancer, and urinary bladder cancer.

[0069] The dendrimers are administered in a dosage dependent on the tumor size and type, location, and other treatments, as well as the agents to be delivered. Typically, an attending physician will decide the dosage of the composition with which to treat each individual subject, taking into consideration a variety of factors, such as age, body weight, general health, diet, sex, compound to be administered, route of administration, and the severity of the condition being treated. In general the timing and frequency of administration will be adjusted to balance the efficacy of a given treatment or diagnostic schedule with the side-effects of the given delivery system. Exemplary dosing frequencies include continuous infusion, single and multiple administrations such as hourly, daily, weekly, monthly or yearly dosing.

[0070] It will be understood by those of ordinary skill that a dosing regimen can be any amount and for any length of time sufficient to treat a brain tumor to reduce size, metastasis, or rate of growth, or to alleviate one or more symptoms such as swelling, pain, or seizures. Physicians routinely determine the length and amounts of therapy to be administered.

B. Adjunct or Combination Therapies

[0071] The dendrimer complexes can be administered in combination with one or more additional therapeutically active agents, which are known to be capable of treating brain tumors or the symptoms associated therewith.

[0072] For example, the dendrimers may be administered to the brain via intravenous administration or during surgery to remove all or a part of the tumor. The dendrimers may be used to deliver chemotherapeutic agents,

immunotherapeutic agents, agents to enhance adjunct therapy such as of a subject undergoing radiation therapy, wherein the poly(amidoamine) (PAMAM) hydroxyl-terminated dendrimers are covalently linked to at least one radiosensitizing agent, in an amount effective to suppress or inhibit the activity of DDX3 in the proliferative disease in the brain.

[0073] It will be understood by those of ordinary skill in the art, that in addition to chemotherapy, surgical intervention and radiation therapy are also used in treatment of cancers of the CNS. Radiation therapy, as used herein, means administering ionizing radiation to the subject in proximity to the location of the cancer in the subject. In some embodiments, the radiosensitizing agent is administered in 2 or more doses and subsequently, ionizing radiation is administered to the subject in proximity to the location of the cancer in the subject. In further embodiments, the administration of the radiosensitizing agent followed by the ionizing radiation can be repeated for 2 or more cycles.

[0074] Typically, the dose of ionizing radiation varies with the size and location of the tumor, but is dose is in the range of 0.1 Gy to about 30 Gy, preferably in a range of 5 Gy to about 25 Gy.

[0075] In some embodiments, the ionizing radiation is in the form of stereotactic ablative radiotherapy (SABR) or stereotactic body radiation therapy (SBRT).

C. Imaging and Diagnostics

[0076] The dendrimers are also useful in a method for imaging TAM associated with a proliferative disease in a subject. The dendrimers are linked to at least one detectable moiety, administered to the subject intravenously in an amount effective to detect the TAM in the subject.

[0077] The dendrimer compositions can be formulated for theranostic purposes. In other words, the dendrimer compositions can comprise multiple compositions which include at least one biologically active agent and at least one detectable moiety. In some cases the at least one biologically active agent and at least one detectable moiety can be the same molecular entity. Thus, the dendrimer compositions can be used to detect the proliferative

disease or tumor in the body of the subject and deliver a biologically active agent to the tumor or TAM simultaneously.

[0078] The present invention will be further understood by reference to the following non-limiting examples.

Example 1: Administration of Fluorescently Labeled Dendrimer to Gliomas in Rats.

Materials and Methods.

[0079] The following agents were purchased: hydroxyl terminated ethylenediamino-core PAMAM dendrimer (referred to as dendrimer throughout, unless otherwise specified) (Dendritech, Midland, MI), Methanol (HPLC grade), DMF (HPLC grade), stainless steel beads (Fisher Scientific, Waltham, MA); and Cyanine 5 (Cy5) (GE Healthcare Life Science, Pittsburgh, PA). For confocal microscopy: nuclei counterstain, 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI), Alexa Fluor® 594 Goat Anti-Rabbit IgG (H+L) Antibody (Molecular Probes, Eugene, Oregon); Fluorescent mounting media (Dako, Santa Clara, CA); Anti-Iba1, Rabbit (Wako, Osaka, Japan); Lectin from *Bandeiraea simplicifolia* (BSI-B4) (Sigma-Aldrich, St. Louis, MO); Anti-GFAP 488 (eBioscience, San Diego, CA); Fluorescein isothiocyanate-dextran (FITC-dextran), average molecular weight 70,000 (Sigma Aldrich, St. Louis, MO).

Synthesis of dendrimer Cy5 (D-Cy5) conjugates

[0080] D-Cy5 was prepared through two steps following the method of *Mol Pharmaceutics*, 10:4560 (2013). Briefly, hydroxyl-terminated PAMAM dendrimer was surface-modified with amine groups to make a bifunctional dendrimer. 6-(Fmoc-amino)caproic acid was used to produce a Fmoc-protected bifunctional dendrimer intermediate that was eventually de-protected by re-dissolving in piperidine/DMF mixture. Cy5 dye with N-hydroxysuccinimide monoester was reacted with amine groups on the surface of bifunctional dendrimer. The 'crude' products were further extensively purified by dialysis. The final D-Cy5 conjugate was characterized using ¹H NMR, high-performance liquid chromatography (HPLC) and gel permeation chromatography (GPC). The conjugate was

stored as a solid powder at -20 °C and reconstituted at 10 mg/ml with sterile 0.9% NaCl on the day of administration.

Tumor inoculation.

[0081] Female Fischer 344 rats, weighing 125-175 g each (Harlan Bioproducts, Indiana, IN), were housed in standard facilities and given free access to food and water. 9L gliosarcoma intracranial implantation was performed as described in *Neurosurgery* 2010, 66, 530-7; *J. Neurosurg.* 2010, 113, 210-7. Briefly, the 9L gliosarcoma (obtained from the Brain Tumor Research Center, UCSF, San Francisco, CA) was maintained in the flank of F344. Tumor was surgically excised from the flank of the carrier animal, sectioned into 1 mm³ pieces and placed in sterile 0.9% NaCl on ice for intracranial implantation. Rats were anesthetized and a midline scalp incision was made to identify the sagittal and coronal sutures. A burr hole was made 3 mm lateral to the sagittal suture and 5 mm posterior to the coronal suture. The dura was incised, and using a surgical microscope and gentle suction a small cortical area was resected. A tumor piece was placed in the resection cavity and the skin was closed using surgical staples. All animals were treated in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee.

D-Cy5 administration for quantification and immunofluorescence.

[0082] For tail vein injections, animals were immobilized and their tails were heated to induce vasodilation. 3mg/300µl of the dendrimer-Cy5 solution was administered per animal. For imaging of dendrimer and dextran distribution, 3 animals were co-injected with a 0.9% NaCl solution of 2 mg D-Cy5 and 2 mg dextran-FITC in 300µl.

[0083] To study the dynamics of dendrimer accumulation in the tumor brain, D-Cy5 was injected into 27 tumor inoculated rats when the average tumor size was 6mm in diameter and then animals were sacrificed at fixed time points (15 minutes, 1 hour, 4 hours, 8 hours, 24 hours, and 48 hours). Magnetic resonance imaging was used to measure intracranial tumor size. Blood was drawn through cardiac puncture and immediately centrifuged to collect plasma. Brains were harvested and flash frozen on dry ice for

fluorescence spectroscopy based quantification or placed in 4% formalin solution for immunofluorescence.

[0084] To study the dendrimer cell uptake, D-Cy5 injection was performed in 3 tumor inoculated rats and 3 healthy rats, and animals were sacrificed 24 hours after the injection. Brains were harvested and placed in 4% formalin for immunofluorescence study.

[0085] To study the pharmacokinetics and biodistribution of dendrimer in plasma and systemic organs D-Cy5 was injected into 15 tumor-inoculated rats which were placed in metabolic cages for urine collection and animals were subsequently euthanized at fixed time points (15 minutes, 1 hour, 4 hours, 8 hours, 24 hours, and 48 hours). Organs were harvested and flash frozen on dry ice for fluorescence spectroscopy-based quantification or placed in 4% formalin for immunofluorescence.

Fluorescence spectroscopy

[0086] Fluorescence-based quantification of D-Cy5 conjugates followed the protocol in Lesniak, Mol Pharm. 2013 Dec 2;10(12):4560-71. Briefly, 100–150 mg of frozen tissue was homogenized in 1 ml of methanol using a homogenizer (TissueLyser LT, Qiagen) in 2ml DNA LoBind Eppendorf tubes and subsequently sonicated. Suspensions were diluted to 100 mg/ml and centrifuged at 15,000 rpm for 15 minutes at 4 °C. The resulting supernatants were subjected to fluorescence spectroscopy. Importantly, prior studies showed that D-Cy5 was stable in plasma, and could be recovered from the tissue intact, without appreciable release of the conjugated Cy5.

[0087] For the brain tissue, precise dissection of the tumor was performed and the peritumoral area was defined as up to 1 mm away from the tumor dissection plane. In the contralateral hemisphere 100 mg of the caudate/putamen with the surrounding white matter area was dissected and used for analysis. For plasma and urine samples a sample of 100 µl of plasma and urine was mixed with 900 µl of phosphate buffer (0.1M) and analyzed by fluorescence spectroscopy.

[0088] Fluorescence spectra of D-Cy5 conjugates and that obtained from tissue extracts were recorded using a Shimadzu RF-5301 Spectrofluorophotometer (Kyoto, Japan). D-Cy5 calibration curves were

constructed, following every experiment, under different slit widths using the maximum emission wavelength of 662 nm after recording spectra from 650 nm to 720 nm with excitation wavelength of 645 nm. The D-Cy5 concentration was measured in methanol or phosphate buffer (0.1 M) in solutions ranging from 1 ng/ml to 100 µg/ml. The slit width was chosen based on the observed fluorescence level of different sample sets. For biological samples with low levels of D-Cy5 (i.e. brain, lung, heart), the excitation and emission slit width was set at 10; for biological samples with high levels of D-Cy5, (i.e. urine and kidney) excitation and emission slit width was set at 3. For the remaining biological samples, excitation slit width of 5 and emission slit width of 10 were used. All calibration curves exhibited linearity with $R^2 \sim 0.99$. Fluorescence registered from tissue of non D-Cy5 injected healthy and tumor inoculated rats was subtracted from the values observed from samples of D-Cy5 injected tissue in order to account for tissue autofluorescence.

[0089] Concentration of D-Cy5 conjugate in the brain was expressed in µg per g of tissue. The concentration of D-Cy5 conjugate in the other organs was expressed in percentage (%) of injected dose per g of tissue or % of injected dose per organ. Concentrations of the D-Cy5 conjugate in urine and blood were expressed in % of injected dose per ml or % of injected dose in total amount of urine or plasma. Total plasma concentration was calculated based on the weight of the animal (*J. Nucl. Med.*, 1985, 26, 72-6). The brain and plasma quantification data were analyzed to calculate the area under the curve (AUC) and the brain to serum ratio.

[0090] The permeation constant (K_{in}) and the initial volume of distribution (V_i) were calculated in the brain tumor (*Nanomedicine (Lond)* 2013, 9, 111-21; *J. Cereb. Blood Flow Metab.* 1983, 3, 8-32). The brain to serum ratio and the area under the curve of the serum ($AUC_{(serum)}(t)$) to serum concentration ($Serum(t)$) were calculated for each time point and linear regression analysis was performed in order to get the K_{in} and V_i based the following equation:

$$\frac{Brain(t)}{Serum(t)} = K_{in} \frac{AUC_{serum}(t)}{Serum(t)} + V_i$$

[0091] For all three regions in the brain, the multiple time-point regression analysis showed good linearity with R^2 equal to 0.98, 0.99, and 0.96 for the contralateral hemisphere, the peritumoral, and the tumor region, respectively (Figure 3A).

Immunofluorescence

[0092] Freshly harvested tissues were fixed in 4% formalin for 24 hours, followed by a gradient of sucrose solutions before cryosection. Tissues were then sectioned transversely into 30 μm -thick slices using a Leica CM 1905 cryostat. Slices were stained with DAPI (nuclei), rabbit anti-Iba1 antibody for microglia/macrophages, and goat anti-rabbit 595 secondary antibody. Some slices were stained with isolectin for endothelial cell staining. Slices were then imaged using a confocal LSM 710 microscope (Carl Zeiss; Hertfordshire, UK) under 5X, 20X, 40X and 63X magnifications. For each slice of tumor-inoculated brains, images were acquired for the tumor, tumor border and contralateral hemisphere. For control (non-tumor) brains, 1-3 representative images were acquired. Settings were optimized to avoid background fluorescence based on non-injected control rat brains. Laser power, pinhole, gain, offset and digital gain were selected separately for each magnification and kept constant throughout the entire study.

Software

[0093] For image processing Zen software was used, any adjustments in brightness and contrast were kept constant throughout the same magnification images. No adjustments were done on the Cy5 channel. Imaris software was used for cell counting, co-localization and microglia surface to volume ratio measurements. Microsoft Excel 2010 and KaleidaGraph 4.0 were used for all calculations, curve fitting and figure plotting related to the pharmacokinetic study.

Cell count and co-localization

[0100] For microglia/macrophage cell count 20X 13 \times 13 tile scan images were analyzed and 3-5 slices were analyzed per region. The function 'spots' was used to identify Iba1+ microglia/macrophages. A diameter threshold of 4.15 μm was set to eliminate the objects smaller than microglia cells and an

intensity threshold of 26.801 based on 'Quality' analysis was set to eliminate the background signal.

[0101] To study co-localization 40X, 4×4 tile scan images were used and 3-5 slides were analyzed per region. The function 'spots' was used to identify DAPI+ nuclei, Iba1+ microglia/macrophages and D-Cy5+ cells. For cells with D-Cy5 uptake, the spots with D-Cy5 and DAPI co-localization were counted; for microglia cells with D-Cy5 uptake, the spots with DAPI, anti-Iba1 and D-Cy5 co-localization were counted. Estimated diameters were applied to eliminate the spots with size smaller than cells, and signal thresholds were applied based on 'Quality' analysis. The function co-localize spots was used by counting the spots where D-Cy5 signal and cell signal are within 10 μm next to each other.

[0102] For surface to volume ratio analysis of the microglia cells, 3D representation of microglia morphology was acquired in confocal microscope using 40X magnification, with 3×3 tile scan, extending 10 μm in the z direction in z-stack. The function 'surfaces' was used and the individual Iba1+ microglia/macrophages were analyzed for surface and volume of each cell. Tumor area, ipsilateral (non-tumor area), contralateral area, and non-tumor brains were analyzed, approximately 150 cells were included for each region. The threshold settings were based on the diameter of cells (*Nat. Neurosci.* 2009, 12, 872-8).

Statistical analysis

[0103] Statistical analysis of data was carried out by student's t-test and one-way ANOVA followed by Games-Howell tests with SPSS 18.0 (IBM, Inc.), as needed. Differences were considered statistically significant at $p < 0.05$.

Results

Pharmacokinetics of systemically Delivered D-Cy5 in Intracranial Brain Tumor

[0104] Figures 2A and 2B show the pharmacokinetic analysis of D-Cy5 in different regions of tumor bearing rodent brain: tumor, peritumor and contralateral hemisphere. The AUC at 48 hours was listed separately to demonstrate the large difference between tumor and contralateral

hemisphere. Based on fluorescence confocal microscopy, dendrimers rapidly accumulated throughout a 5 mm tumor, as early as fifteen minutes after systemic administration.

[0105] In contrast, in the 'healthy' contralateral hemisphere, dendrimers outlined the blood vessels, and were not observed in the parenchyma using confocal microscopy images of tumor inoculated rodent brain following systemic administration of D-Cy5. Images indicate the homogeneous distribution of D-Cy5 in the tumor and the restriction of dendrimer in the blood vessel lumen. Fifteen minutes after systemic administration, the dendrimer was dispersed throughout the entire intracranial tumor parenchyma. This distribution was not influenced by the heterogeneity in the tumor parenchyma. There was no appreciable cellular uptake at this time point. At 4 hours post systemic dendrimer administration, the extracellular distribution in the tumor region had decreased, which was accompanied by an increased uptake by Iba1+ inflammatory cells. The contralateral hemisphere showed relatively minimal D-Cy5 fluorescence at all the time points.

[0106] In order to assess the kinetics of dendrimer accumulation in the brain, a recently developed fluorescence-based semi-quantification method for D-Cy5 was used. The use of the near IR Cy5 wavelength overcomes the tissue autofluorescence challenges. The high sensitivity of this method (0.1 ng/g of tissue) allowed detection of dendrimer accumulation at specific anatomic locations. In accordance with the confocal microscopy results, dendrimer rapidly accumulated in the tumor and peritumoral area, with a peak concentration occurring at 8 hours. See Figure 3A. Dendrimer gradually cleared from the tumor at a rate of $\sim 0.01 \mu\text{g/g/hour}$, and from the peritumoral area at a rate of $\sim 0.007 \mu\text{g/g/hour}$, reaching a concentration of $0.2 \mu\text{g/g}$ of tissue, 48 hours after initial systemic injection. In the contralateral hemisphere, the dendrimer accumulation also peaked at 8 hours, at a concentration ~ 8 -fold lower than that found in the tumor area. At 24 hours, traces of dendrimer ($0.03 \mu\text{g/g}$) could be detected in the contralateral hemisphere and a ~ 14 fold higher accumulation in the tumor was observed. At 48 hours the AUC was 10 times higher in the tumor area in comparison to

the contralateral hemisphere, indicating significantly higher ($p < 0.05$) overall exposure of the dendrimer to the tumor. The high and selective retention of dendrimer in the tumor and peritumoral area was visualized in a low magnification image of the tumor stained for astrocytes 24 hours following the administration of dendrimer.

[0107] The vasculature and the amount of BBTB disruption have been demonstrated to differ significantly between the tumor core and a tumor border which may play a significant role in drug accumulation. Therefore, blood vessel endothelial cells were stained in order to examine the difference in vasculature and, therefore, perfusion between the tumor and the peritumoral area. As expected, the peritumoral area and the tumor border showed dramatically denser vasculature than the tumor core. However, the dendrimer distribution appeared to be uniform in the tumor.

[0108] To further understand the kinetics of dendrimer penetration in the tumor the permeation constant (K_{in}) and the initial volume of distribution (V_i) in the tumor, peritumoral area and contralateral hemisphere, were calculated. See Figure 3B. K_{in} describes the influx of dendrimer from the blood to the brain, and was 10 fold higher in the tumor and peritumoral area comparison to the contralateral hemisphere, indicating the increased penetration of dendrimers and increased permeability and perfusion of the tumor tissue. V_i represents the volume of the brain compartments in rapid equilibrium with plasma, differed significantly between the tumor and the peritumoral area indicating a larger volume of rapid equilibration in the tumor core. In tumor xenografts the hypoxic tumor core has increased vascular permeability in comparison to the tumor border and in glioblastoma specifically a distinct difference in the morphology of the BBTB between the tumor core and the peritumoral area has been shown, which may contribute to the rapid distribution in the tumor core as opposed to the peritumoral area.

Bio-distribution of D-Cy5: Imaging-Based Study in Intracranial Brain Tumor

[0109] To study the dendrimer distribution in the tumor and peritumoral area, D-Cy5 was co-injected with linear dextran-FITC (70 kDa, ~6.5 nm radius) which has approximately twice the size of dendrimer. The tumor was

clearly identified based on the increased density of DAPI-positive nuclei. See Figures 4A and 4B. At each time point, the dendrimer distributed homogeneously throughout the whole tumor region and a markedly decreased signal was observed 24 hours after injection. See Figure 5. In comparison, signal from dextran-FITC was only observed around the tumor border and not in the tumor core, even when the laser power and gain settings were increased to see the background signal in the FITC channel. Higher magnification images showed that dendrimer rapidly distributed and delineated the extracellular matrix (ECM) leading to a reticular pattern of distribution and gradual accumulation in the cells. On the contrary, dextran showed limited distribution throughout the extracellular matrix but high signal could be seen within the blood vessel lumen fifteen minutes after injection. At later time points, limited amounts of dextran were retained in the tissue presumably due to low cellular uptake.

Characterization of Tumor Associated Microglia/Macrophages and Cell Uptake of Dendrimer

[0110] Gliomas produce chemo-attractants and growth factors that promote recruitment and proliferation of microglia/macrophages. In human glioblastoma up to 30% of cells can be tumor associated macrophages. The microglial distribution was determined in different anatomic locations of the 9L tumor model, which suggested that the concentration of TAM in this tumor model is similar to that seen in human glioblastoma. The microglia population per mm² was 9-fold higher within the tumor as compared to the contralateral hemisphere and 2.5-fold higher in healthy brain tissue of the ipsilateral hemisphere as compared to the contralateral

[0111] TAM is reprogrammed in the tumor microenvironment, leading to an alternate immunosuppressive M2 phenotype. However, a number of studies have suggested sustained phagocytic activity of TAM in glioma. The phagocytic activity of TAM has been suggested to play a key role in nanoparticle uptake. When microglia/macrophages change from a resting to an activated form, their morphology is modified from ramified to amoeboid indicative of their increased phagocytic activity. In order to assess the morphology of TAM in the 9L tumor model the surface to volume ratio of

the immune cell population (Iba1+) in different anatomical locations was characterized. The surface to volume ratio (StoV ratio) is considered a 'measure' of microglial activation. The results indicate that the microglia/macrophages within the tumor and surrounding the tumor had a significantly ($p < 0.001$) lower StoV ratio in comparison to the immune cells in the contralateral hemisphere, and in a healthy brain. The mean StoV ratio for tumor associated microglia was lower than 1, indicative of their amoeboid state and phagocytic activity. Image based cell count of the Iba1+ microglia/macrophages population per mm^2 area in the tumor, ipsilateral hemisphere and contralateral hemisphere. Image based measurement of microglia cell surface to volume ratio as an indication of activation and phagocytic activity of microglia/macrophages in healthy brain, contralateral hemisphere and ipsilateral hemisphere of a tumor inoculated brain and tumor tissue. 1+ cells D-Cy5 co-localization with Iba1+ TAMs and D-Cy5 co-localization with DAPI+ cells. Results are expressed as percent of the total DAPI+ cell population. There is no statistical significance between microglia uptake and cell uptake. Statistical * $p < 0.05$; ** $p < 0.001$ Statistical analysis is based on 3-5 different slices. See Figures 4A and 4B.

[0112] The localization of D-Cy5 in the Iba1+ microglia/macrophages cells was then calculated. At 24 hours post systemic administration, dendrimers localized in the Iba1+ TAM. Iba1+ microglia/macrophages were calculated to comprise 38% of the total tumor cell population (Figure 5). Co-localization indicated that approximately half of the TAM population took up dendrimers and that the total population of dendrimer-positive cells did not differ quantitatively from the population of dendrimer-positive Iba1+ microglia/macrophages. Therefore, dendrimers were taken up almost exclusively by tumor associated macrophages within the tumor tissue, while other cells within the tumor region did not have measurable dendrimer uptake. In the tumor border (1 mm from the tumor edge based on DAPI stain) the dendrimer-positive microglia/macrophages were substantially reduced, reflecting the difference in the biological processes between the tumor core and the tumor border. Dendrimer was not present in the ipsilateral non-tumor region or in the contralateral hemisphere.

[0113] D-Cy5 cell localization analysis 24 hours following administration was performed using image based measurement of Iba-1+ cells D-Cy5 co-localization with Iba1+ TAMs and D-Cy5 co-localization with DAPI+ cells. Results are expressed as percent of the total DAPI+ cell population. There is no statistical significance between microglia uptake and cell uptake. High magnification (40X) fluorescence confocal imaging of different anatomic locations of a 9L gliosarcoma inoculated brain.

Systemic Biodistribution of D-Cy5

[0114] After 24 hours systemic administration, fluorescence-quantification of extracted D-Cy5, suggested that 56% of the dendrimer was excreted through the urine, while 32% remained in the kidneys. This correlates well with the low serum levels (0.66%, Figure 6A) at this same time point. After 24 hours systemic administration, only 2.5% of the dendrimers were accumulated in other major organs. 1.5% of these dendrimers were retained in the liver and spleen representing the elimination by the reticuloendothelial system (RES). Trace amounts accumulated in the brain, lung, and heart.

[0115] To better understand the kinetics, dendrimer concentration in the serum and major organs was measured over time (Figure 6B). The kidney had an increasing accumulation at all chosen time points up to 24 hours. After 48 hours from systemic administration, the amount of dendrimer in the kidneys began to decrease. Dendrimer serum level decreased rapidly with only 4% of the injected dose observed 15 minutes after systemic injection (Figure 8A).

[0116] Figure 6A shows the fluorescence based quantification of D-Cy5 in major organs (brain, liver, lung, spleen, heart, and kidney), serum and urine of 9L gliosarcoma rodent model 24 hours following D-Cy5 administration. The biodistribution is expressed in percent of injected dose per organ; (inserted panel): D-Cy5 accumulation in kidney, urine and other organs. (6B) Time dependent concentration of D-Cy5 in spleen, liver, kidney and serum. Concentration is expressed in percent of injected dose per g of tissue. (57C) Fluorescence based quantification of the plasma pharmacokinetics of D-Cy5.

Renal Accumulation and Distribution

[0117] The high concentration of dendrimer in the renal system led to the study of the distribution in the kidney in order to assess the sites of accumulation. Based on fluorescence microscopy, the dendrimer accumulated in the renal cortex. Dendrimer co-localized with anti-GFAP antibody which stained the peritubular fibroblasts. No presence of dendrimer was observed in the glomeruli. There was no significant difference in terms of the clearance through the kidneys among healthy and tumor-bearing rats.

Summary and Conclusions

[0118] Malignant glioma is the most common primary brain tumor and results in more years of life lost than any other tumor. Numerous traditional small molecule chemotherapeutic drugs in preclinical and clinical trials fail to have a drastic impact in the natural history of the disease due to their low accumulation and rapid clearance from the tumor following systemic administration.

[0119] At 48 hours the dendrimer accumulation in the brain tumor is 11 fold higher than that in the contralateral hemisphere. This ratio stands out in comparison to other non-targeted nanoparticles and is comparable to actively targeted magnetic nanoparticles. Importantly, high retention is also observed in the peritumoral area. Glioblastoma cells are highly infiltrative and can be found in anatomic locations with intact blood brain barrier; thus achieving high retention at the peritumoral area is of distinct importance for the design of an effective therapeutic vehicle. Selective retention of dendrimers in the tumor and peritumoral area in combination with low circulation half-life allows for specific delivery of chemotherapeutics with limited off target effects.

Example 2: Hydroxyl terminated generation 6 PAMAM dendrimers as therapeutic vehicles for the treatment of glioblastoma

Materials and Methods

[0120] The materials and methods are as described in Example 1, with the exception that both generation 4 and 6 hydroxyl terminated PAMAM dendrimers were utilized.

[0121] To study the dynamics of dendrimer accumulation in the tumor brain, dendrimers were injected into tumor inoculated rats when the average tumor size was 6mm in diameter and then animals were sacrificed at different time points.

Results

[0122] The efficacy of chemotherapeutics is directly associated with the amount of accumulation in the tumor. Increasing the dendrimer size from generation 4 to generation 6 increased their hydrodynamic diameter from ~4.3nm to ~6.7nm without significantly influencing their ζ -potential (Table 1). Hydrodynamic diameter (size) and surface charge (ζ -potential) were measured using dynamic light scattering in PBS, pH 7.4 at room temperature. Molecular weight was provided by the supplier.

Table 1: Physiochemical properties of hydroxyl terminated dendrimers with generation 4 (G4-OH) and generation 6 (G6-OH).

Dendrimer MW (kDa) Size + SEM (nm) ζ -potential + SEM (mV)

G4-OH	14.1	4.3 \pm 0.2	+4.5 \pm 0.1
G6-OH	58.0	6.7 \pm 0.1	0.25 \pm 0.4

[0123] Example 1 shows that G4 dendrimers selectively accumulate in TAMs when administered systemically in 9L gliosarcoma inoculated rats at 24hr post D-Cy5 administration. However, the increase of size allowed G6 dendrimers to avoid the rapid clearance caused by renal filtration and to circulate longer in the blood, allowing a better localization and retention. G6 dendrimers showed higher serum concentration and prolonged serum half-life than G4 dendrimers, which contributed to the higher tumor accumulation and targeting of G6 dendrimers.

[0124] Figures 7A-7C are graphs of the comparison of accumulation (μg of dendrimer / g of brain tissue) (Figure 7A) between G6 and G4 dendrimers in a tumor bearing brain as a function of time. The accumulation of G4 dendrimers in the tumor/peritumor peaked around 8 hours post injection, and gradually decreased, while G6 dendrimers concentration in tumor/peritumor continuously increased. At 48 hours, G6 concentration was almost 100-fold

higher than G4 dendrimers concentration in the tumor; the area under curve (AUC) plot (Figure 7B) which demonstrates G6 dendrimers have around 100 fold higher brain tumor exposure than G4 dendrimers within 48 hours.

[0125] When the dendrimer concentration in the brain is normalized by the dendrimer concentration in the serum, the brain to serum ratio is an indication of the brain targeting ability. G6 dendrimers showed higher tumor targeting ability than G4 dendrimers at all the time points (Figure 6C). The dendrimer concentration was demonstrated as percentage of total injected dose per milliliter of serum. For G4 dendrimers, kidney had the most dendrimer accumulation (20%-30%), significantly higher than dendrimer accumulation in liver and spleen (~0.3%) at different time points. For G6 dendrimers, the increase of size greatly decreased the renal filtration and kidney accumulation. The kidney concentration of G6 dendrimers was more than 10 fold less than G4 dendrimers (~1%), and started to show the evidence of clearance from kidney starting from 48 hours. The liver accumulation of G6 dendrimers was similar to G4 dendrimers, while spleen showed ~5 fold higher accumulation, possibly due to the increased uptake by monocytes. The high serum concentration provides a driving force for G6 dendrimers to diffuse across the blood-brain tumor barrier and better target tumor while accumulate in the tumor with 100-fold higher concentration and AUC (at 48 hours) compared with G4 dendrimers (Figure 7B).

[0126] The high frequency of glioblastoma recurrence occurs from the individual cells that survive the aggressive treatment. For this reason it is important that any therapeutic vehicle be able to reach every tumor cell. Recent studies in the nanotechnology field have underlined that efficient distribution of nanoparticles throughout the tumor tissue as a prerequisite for efficacy. Most of these studies revealed that nanoparticles with a size range between 10-50nm could uniformly distribute through the solid tumor, when administered into the tumor. Larger nanoparticles with size of sub-100nm, are less likely to distribute homogeneously in the whole tumor bed, due to the fibrotic tissue, where no blood vessel exist, interweaving in between the nest of cancer cells forms diffusion barrier against the homogeneous distribution of molecules. However, the homogeneous distribution of

nanoparticles also requires them to uniformly extravasate from vasculature without any hindrance. In the case of glioblastoma, this requirement further decreased the upper limit for size cutoff to 10nm, considering the smaller fenestration and pore size in cranial tumors compared with subcutaneous tumor. Unhindered diffusion through the BBTB has been achieved with molecules of 7nm.

[0127] The charge density also influences the penetration of nanoparticles. It is important for nanoparticles to maintain neutral or slightly positive charge instead of strongly cationic to prevent the electrostatically derived binding-site barrier effect. As a result, G4 OH PAMAM dendrimers with hydrodynamic diameter of 4.3nm and neutral surface charge can rapidly distribute through the intervascular spaces in 15mins and homogeneously cover the entire 5mm tumor. Increasing the generation to G6 not only retains the dendrimers' ability to homogeneously distribute in the brain tumor, but also prevents dendrimers from clear out from tumor parenchyma rapidly.

[0128] G4 and G6 dendrimers were co-injected intravenously into the tumor bearing rats through tail vein. The brain was fixed and cryo-sectioned axially. See Figures 8A and 8B. Figures 8A and 8B are graphs showing the G4 and G6 dendrimers concentration in serum and major organs: kidney, liver, spleen as a function of time. Figure 8A shows that G6 dendrimers showed higher serum concentration and prolonged serum half-life than G4 dendrimers, which contributed to the higher tumor accumulation and targeting of G6 dendrimers. The dendrimer concentration was demonstrated as percentage of total injected dose per milliliter of serum. The liver accumulation of G6 dendrimers was similar to G4 dendrimers, while spleen showed ~5 fold higher accumulation, possibly due to the increased uptake by monocytes.

[0129] G4 dendrimers had a faster excretion rate than G6 dendrimers. Figure 8B showed that for G4 dendrimers, kidney had most dendrimer accumulation (20%-30%), significantly higher than dendrimer accumulation in liver and spleen (~0.3%) at different time points. For G6 dendrimers, the increase of size greatly decreased the renal filtration and kidney accumulation. The kidney concentration of G6 dendrimers was more than 10

fold less than G4 dendrimers (~1%), and started to show clearance from kidney starting from 48 hours. For G4 dendrimer, excretion from the blood vessel to tumor tissue started immediately (~15min) after i.v. injection, followed with clearance from tumor after 8 hours. After 24 hours post injection, G4 dendrimers were retained in the cells. For G6 dendrimers, the presence of dendrimers in the tumor tissue was not observed immediately after administration. The concentration of G6 dendrimers gradually increased in the whole observation period. At 48 hours post administration, G6 dendrimers showed the highest concentration in the tumor and peritumor area. Their homogeneous distribution in glioblastoma including the tumor cell migrating front and their retention in the tumor for at least 48 hours confirms the desirability of the dendrimers for efficient delivery of therapeutics.

[0130] In addition to homogeneous distribution across the tumor bed upon systemic administration, dendrimers can also accumulate in the peritumoral region, where deep, active perivascular and perineural invasion of glioma cells into normal nervous tissue occurs. Various techniques have been applied for the targeting of peritumoral region such as the using of GFAP specific monoclonal antibodies to modify the nanoparticles' surface property and the using of magnetic field to guide the accumulation of magnetic nanoparticles. For dendrimers, without any modification or addition of targeting ligands, the area under curve (AUC) in peritumoral region reaches 60% of that in the tumor region, indicating the ability of dendrimers to intrinsically target peritumoral area.

[0131] Finally, dendrimers target neuroinflammatory cells and largely as well as exclusively localize in TAMs while resting ramified microglia in the non-tumor region do not take up dendrimers. Dendrimer specific accumulation in TAMs with low accumulation in the reticuloendothelial system and rapid clearance from the circulation provides a unique advantage for the delivery of anti-glioma therapies targeting TAMs.

[0132] Modifications and variations of the methods and materials described herein will be apparent to those skilled in the art and are intended to be encompassed by the claims.

We claim:

1. A method for treating a proliferative disease in a subject comprising administering to the subject systemically, a composition comprising poly(amidoamine) (PAMAM) hydroxyl-terminated dendrimers covalently linked to or complexed with at least one therapeutic, prophylactic or diagnostic agent, in an amount effective to suppress or inhibit one or more symptom of the proliferative disease, or to image the proliferative disease, in the subject.
2. The method of claim 1 wherein the dendrimers are generation 4-10 poly(amidoamine) (PAMAM) hydroxyl-terminated dendrimers covalently linked to at least one therapeutic agent.
3. The method of claim 1, wherein the PAMAM dendrimer is a generation 6 PAMAM dendrimer.
4. The method of any of claims 1-3 wherein the dendrimer is conjugated to a first therapeutic agent and a second agent selected from the group consisting of therapeutic agents, prophylactic agents, and diagnostic agents.
5. The method of claim 4 wherein the dendrimer is conjugated to two therapeutic agents.
6. The method of claim 5 wherein the dendrimer is conjugated to a chemotherapeutic and an anti-inflammatory and/or an anti-excitotoxicity agent.
7. The method of any of claims 1-6, wherein the dendrimer complex includes a therapeutically active agent for localizing and targeting microglia and astrocytes.
8. The method of any of claims 1-7, wherein the proliferative disease is in the brain of the subject.
9. The method of any of claims 1-8 wherein the dendrimers conjugated to therapeutic agent are in a unit dosage in an amount effective to alleviate the proliferative disorder.
10. The method of any of claims 1-9, wherein the agent is selected from the group consisting of peptides, proteins, carbohydrates, nucleotides or oligonucleotides, small molecules, and combinations thereof.

11. The method of any of claims 1-10, wherein the agent is selected from the group of chemotherapeutic agents consisting of alkylating agents, angiogenesis inhibitors, modulators of tumor immune response, aromatase inhibitors, antimetabolites, anthracyclines, antitumor antibiotics, platinum compounds, topoisomerase inhibitors, radioactive isotopes, radiosensitizing agents, checkpoint inhibitors, PD1 inhibitors, APRKinase inhibitors, plant alkaloids, glycolytic inhibitors and prodrugs thereof.

12. The method of claim 11, wherein the agent is selected from the group of chemotherapeutic agents consisting of taxols such as paclitaxel, BCNU, camptothecin, doxycycline, cisplatin, and derivatives, analogues and prodrugs thereof.

13. The method of any of claims 1-12, wherein at least one agent complexed with or conjugated to the dendrimer is an agent which alleviates one or more symptoms of the tumor and is selected from the group consisting of anti-inflammatory agents, anti-excitotoxicity agents, immunosuppressive agents, anti-angiogenic agents and anti-infectives.

14. The method of claim 13 wherein the anti-inflammatory agent is selected from the group consisting of steroidal anti-inflammatories, non-steroidal anti-inflammatory agents, gold compound anti-inflammatory agents, immunosuppressive agents, salicylate anti-inflammatory agents, ranibizumab, minocycline, and rapamycin.

15. The method of claim 14 wherein the agent which alleviates one or more symptoms of the tumor is an agent having activity as anti-excitotoxic agents and is selected from the group consisting of valproic acid, D-aminophosphonovalerate, D-aminophosphonoheptanoate, inhibitors of glutamate formation/release, NMDA receptor antagonists can also be administered.

16. The method of any of claims 1-15 comprising diagnostic agents selected from the group consisting of paramagnetic molecules, fluorescent compounds, magnetic molecules, radionuclides, x-ray imaging agents, contrast media, fluorescent dyes, Near infra-red dyes, SPECT imaging agents, PET imaging agents and radioisotopes, wherein the

composition is administered to an individual to diagnose information regarding the proliferative disorder in the individual.

17. The method of 16, wherein the dye is selected from the group consisting of carbocyanine, indocarbocyanine, oxacarbocyanine, thüicarbocyanine and merocyanine, polymethine, coumarine, rhodamine, xanthene, fluorescein, boron~dipyrrromethane (BODIPY), Cy5, Cy5.5, Cy7, VivoTag-680, VivoTag-S680, VivoTag-S750, AlexaFluor660, AlexaFluor680, AlexaFluor700, AlexaFluor750, AlexaFluor790, Dy677, Dy676, Dy682, Dy752, Dy780, DyLight547, Dylight647, HiLyte Fluor 647, HiLyte Fluor 680, HiLyte Fluor 750, IRDye 800CW, IRDye 800RS, IRDye 700DX, ADS780WS, ADS830WS, and ADS832WS.

18. The method of claim 16, wherein the SPECT or PET imaging agent is a chelator including di-ethylene tri-amine penta-acetic acid (DTPA), 1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetraacetic acid (DOTA), di-amine dithiols, activated mercaptoacetyl-glycyl-glycyl-gylcine (MAG3), and hydrazidonicotinamide (HYNIC).

19. The method of claim 16 wherein the radioisotopes are selected from the group consisting of Tc-94m, Tc-99m, In-111, Ga-67, Ga-68, Gd³⁺, Y-86, Y-90, Lu-177, Re-186, Re-188, Cu-64, Cu-67, Co-55, Co-57, F-18, Sc-47, Ac-225, Bi-213, Bi-212, Pb-212, Sm-153, Ho-166, and Dy-i66.

20. The method of any of claims 1-19 wherein the dendrimer is complexed with or conjugated to targeting moieties.

21. The method of any of claims 1-20 for treating a proliferative disease in a subject undergoing radiation therapy comprising administering to the subject systemically dendrimers covalently linked to at least one radiosensitizing agent, in an amount effective to suppress or inhibit the activity of DDX3 in the proliferative disease in the subject.

22. The method of claim 21, wherein the radiosensitizing agent is administered in two or more doses and subsequently, administering ionizing radiation to the subject in proximity to the location of the cancer in the subject, optionally repeating administration of the radiosensitizing agent followed by the ionizing radiation for 2 or more cycles.

23. The method of any of claims 21 to 22, wherein the ionizing radiation dose is in the range of 0.1 Gy to about 30 Gy, preferably in a range of 5 Gy to about 25 Gy.

24. The method of any of claims 21 to 23, wherein the radiation is stereotactic ablative radiotherapy (SABR) or stereotactic body radiation therapy (SBRT).

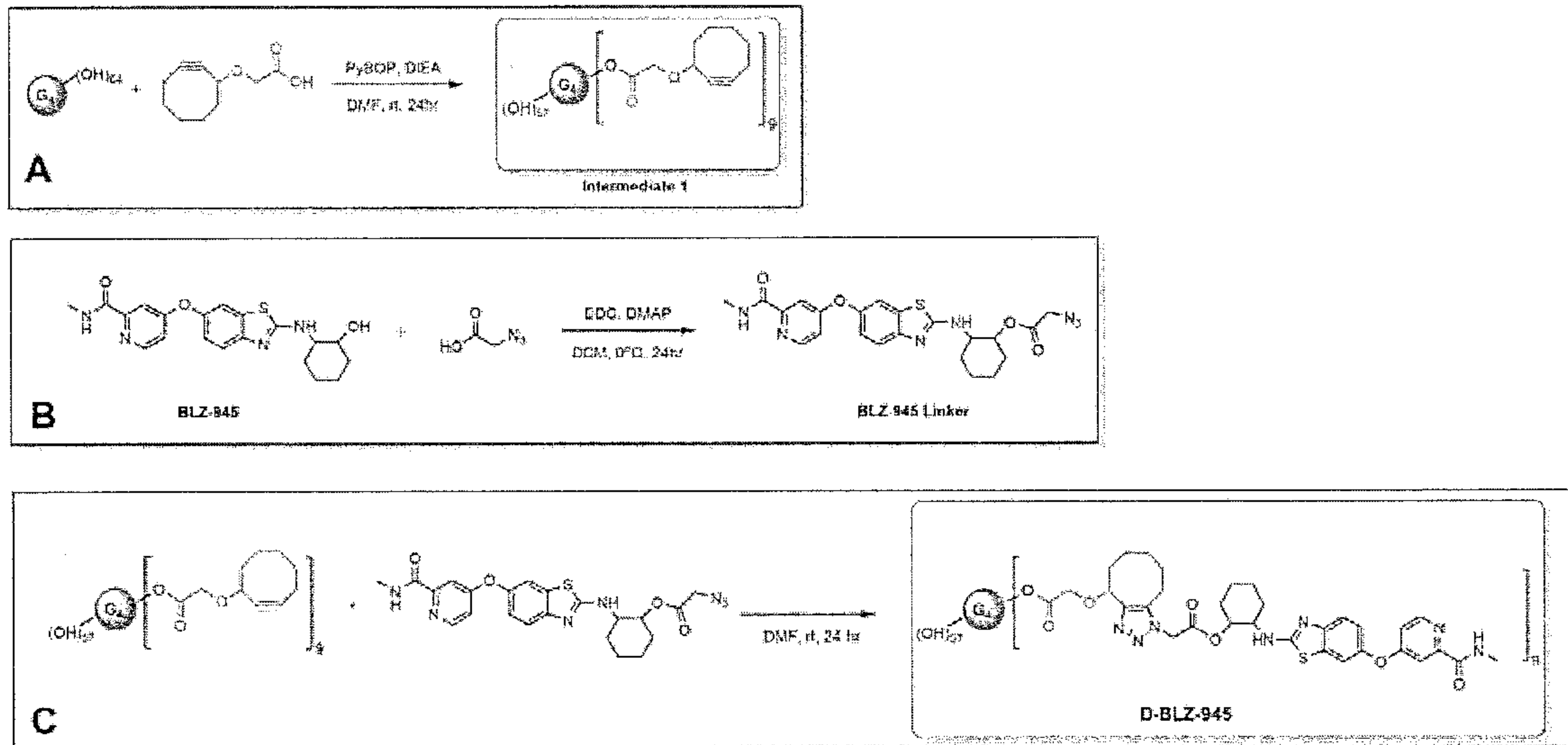
25. The method of any of claims 21 to 24, wherein the radiosensitizing agent is RX-33.

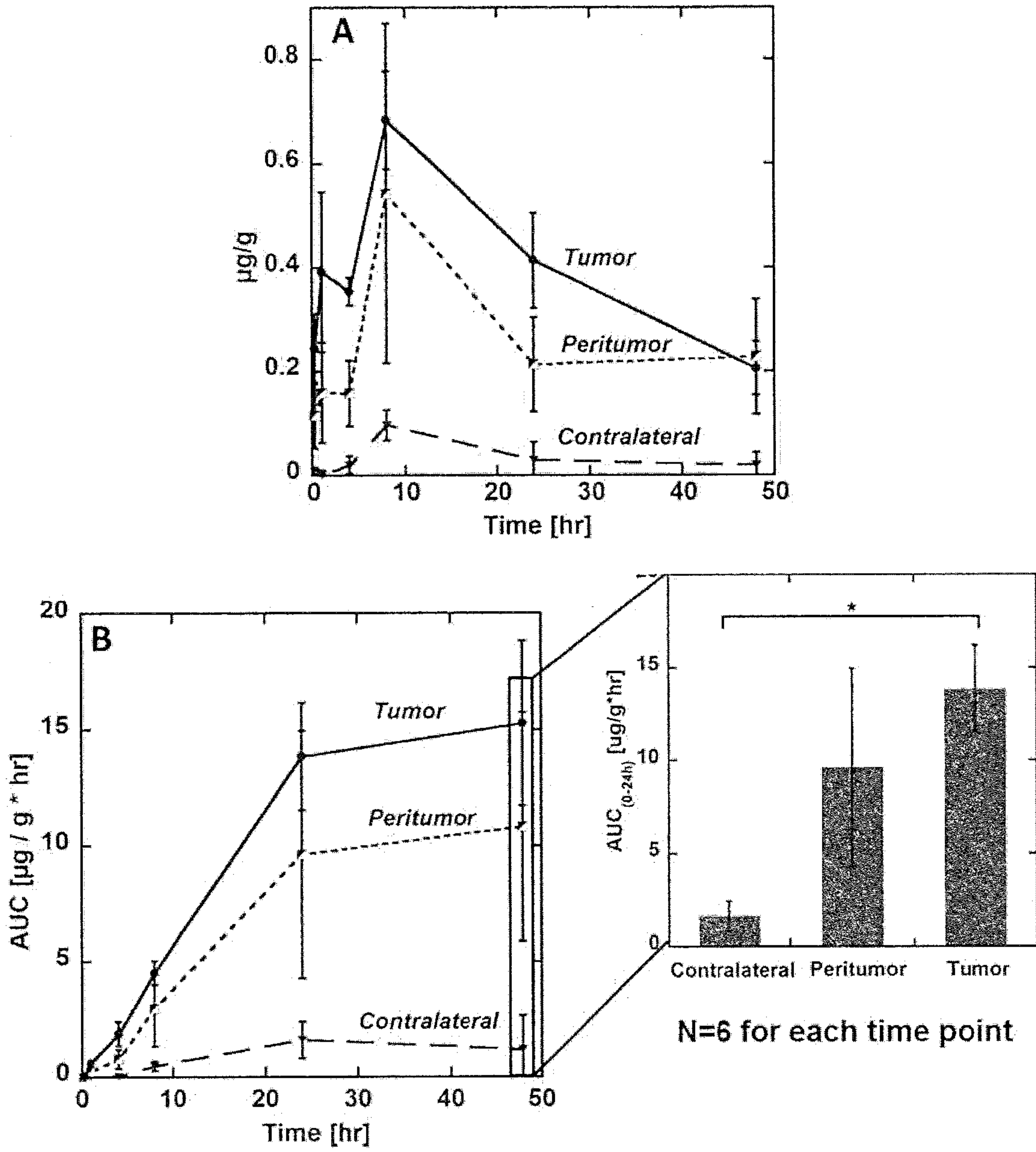
26. The method of any of claims 1-25, wherein the proliferative disease is a solid cancer.

27. The method of claim 26, wherein the cancer originates as alveolar rhabdomyosarcoma, bone cancer, brain cancer, breast cancer, cancer of the anus, anal canal, or anorectum, cancer of the eye, cancer of the intrahepatic bile duct, cancer of the joints, cancer of the neck, gallbladder, or pleura, cancer of the nose, nasal cavity, or middle ear, cancer of the oral cavity, cancer of the vulva, colon cancer, esophageal cancer, cervical cancer, gastrointestinal carcinoid tumor, Hodgkin lymphoma, hypopharynx cancer, kidney cancer, larynx cancer, liver cancer, lung cancer, malignant mesothelioma, melanoma, multiple myeloma, nasopharynx cancer, non-Hodgkin lymphoma, ovarian cancer, pancreatic cancer, peritoneum, omentum, and mesentery cancer, pharynx cancer, prostate cancer, rectal cancer, renal cancer (e.g., renal cell carcinoma (RCC)), small intestine cancer, soft tissue cancer, stomach cancer, testicular cancer, thyroid cancer, ureter cancer, and urinary bladder cancer.

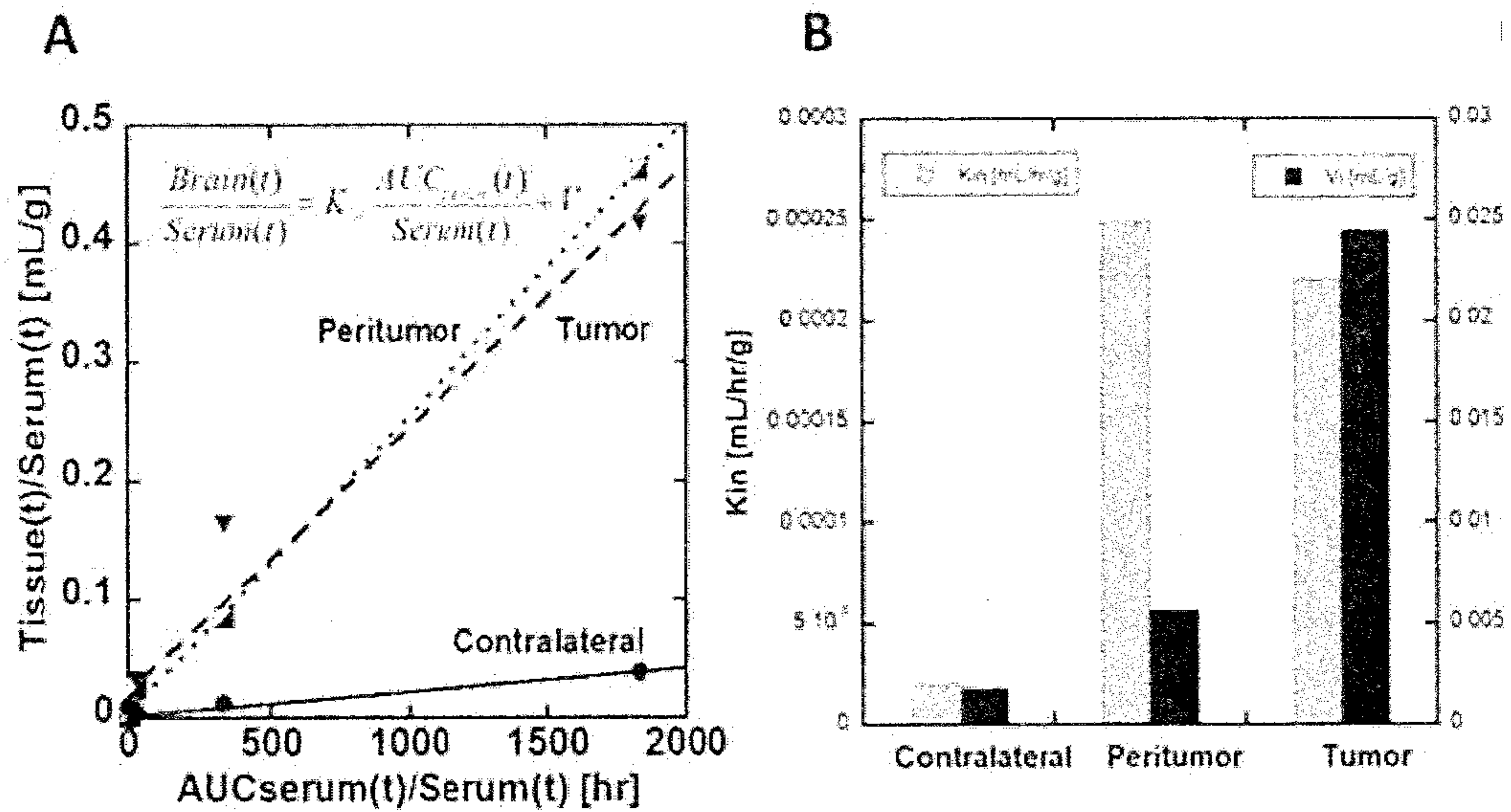
28. The method of claim 26, wherein the proliferative disease is brain cancer selected from the group consisting of neoplasias, hyperplasias and cancers of the central nervous system including gliomas, glioblastoma, astrocytoma, oligodendroglioma, ependymoma, meningioma, medulloblastoma, ganglioma, Schwannoma, craniopharyngioma, cordomas and pituitary tumors.

29. The method of claim 28 wherein the cancer is a glioma.
30. The method of any of claims 1-29, wherein the dendrimer composition is formulated with liposomes, particles, suspensions, solutions or emulsions.
31. The method of any of claims 1-30, wherein the composition is administered to the subject in a time period selected from the group consisting of: daily, weekly, biweekly, monthly, and bimonthly.
32. A formulation for use in the method of any of claims 1-31.





FIGs. 2A-2B



FIGs. 3A-3B

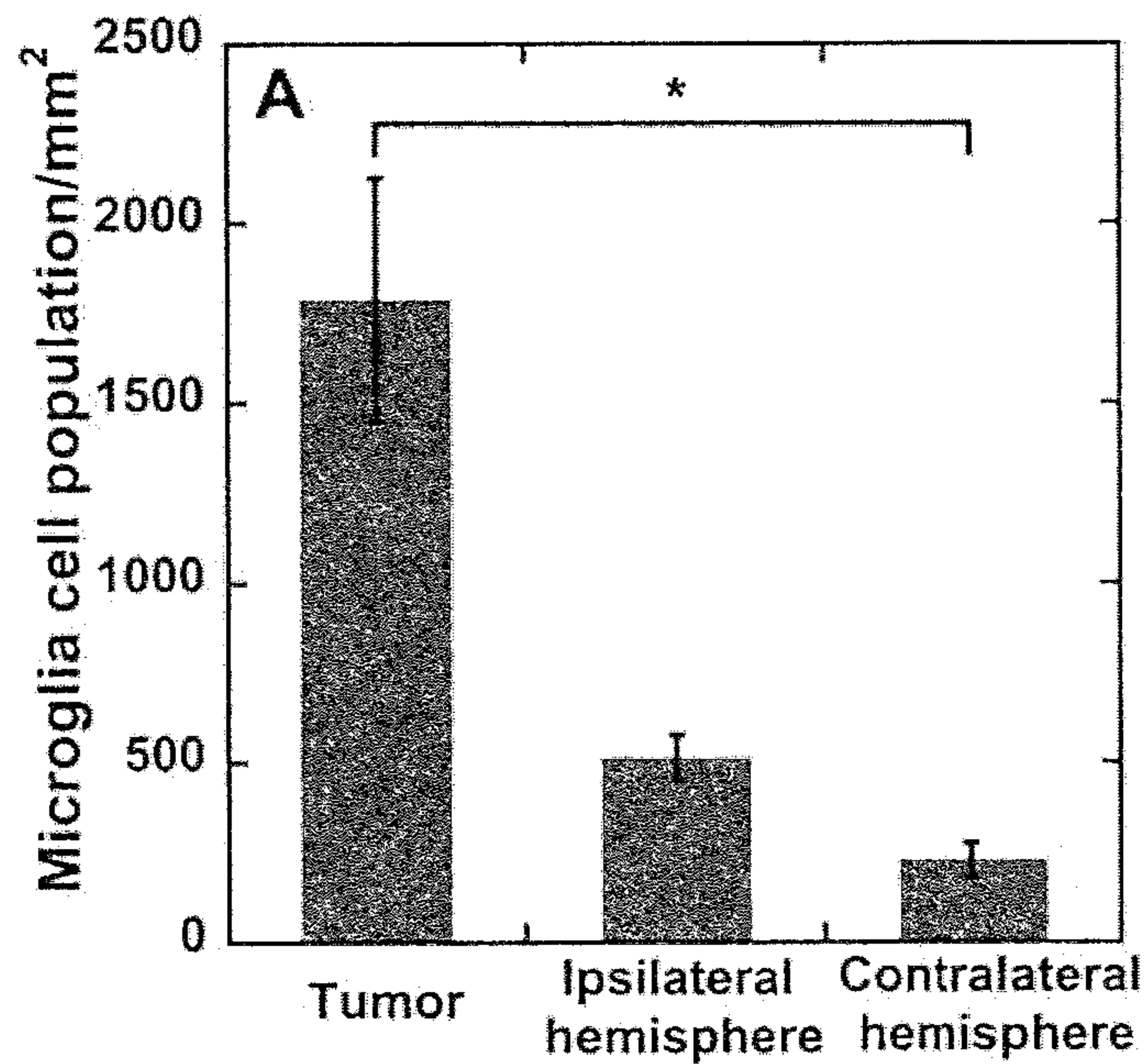


FIG. 4A

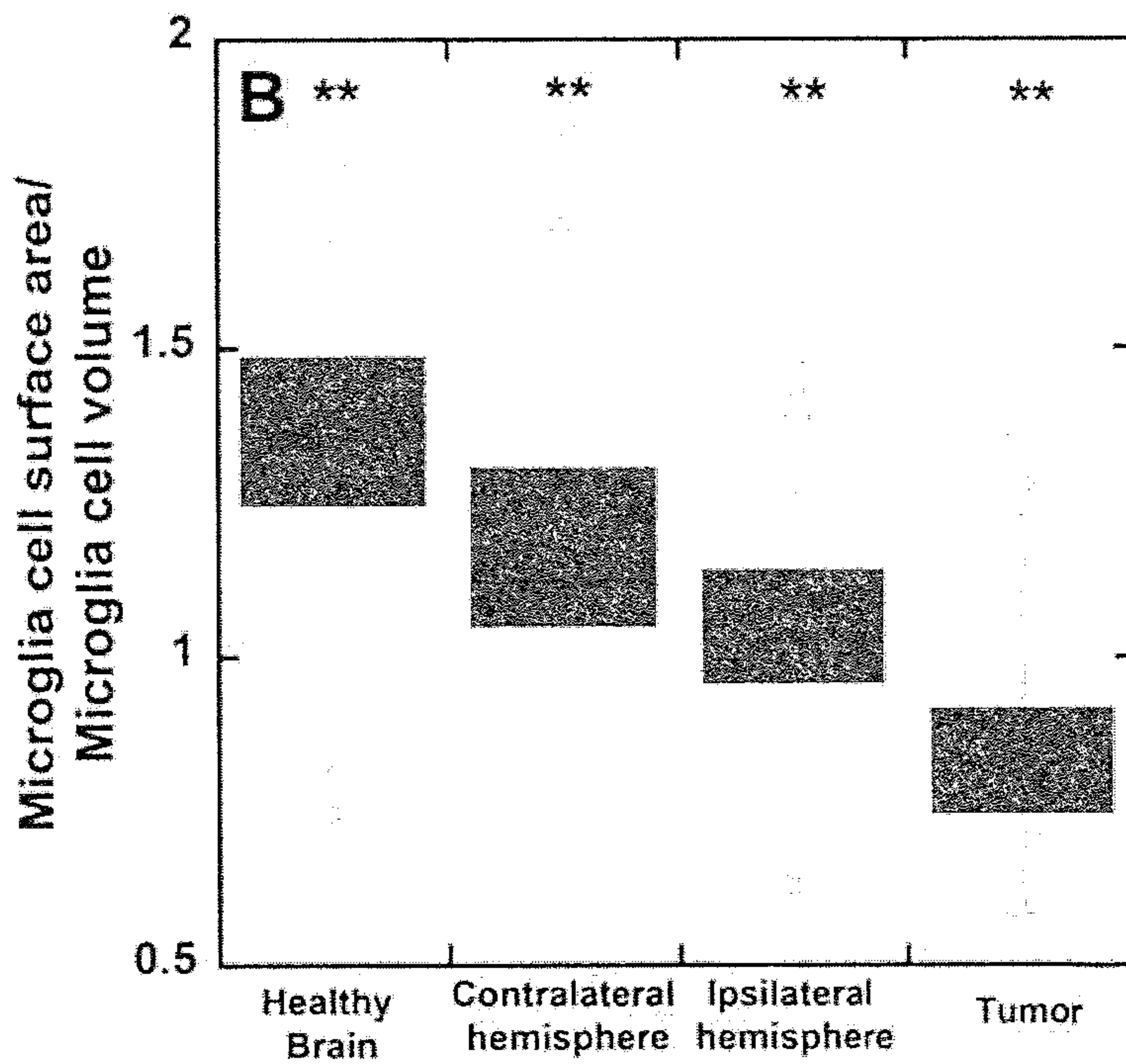


FIG. 4B

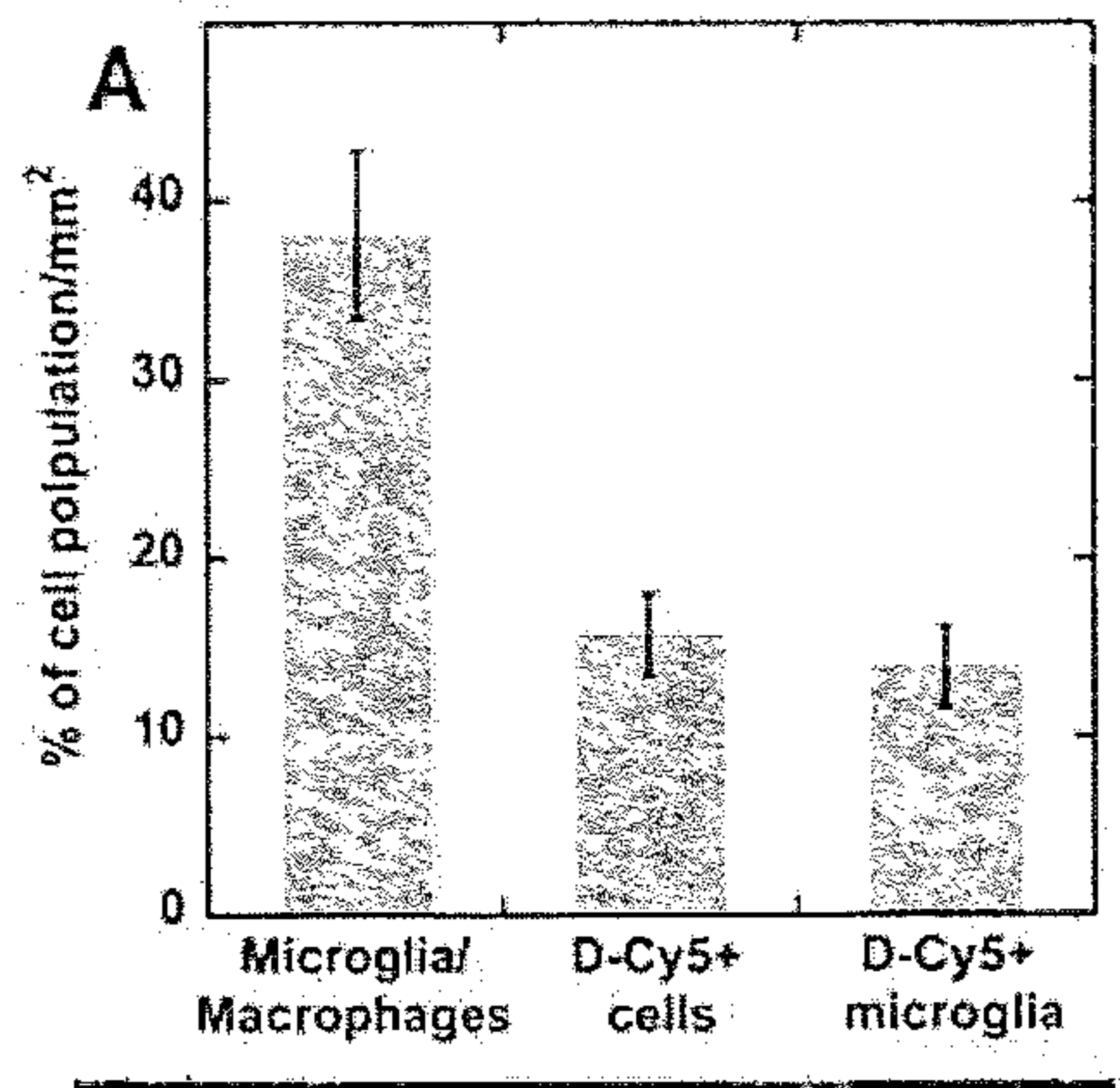
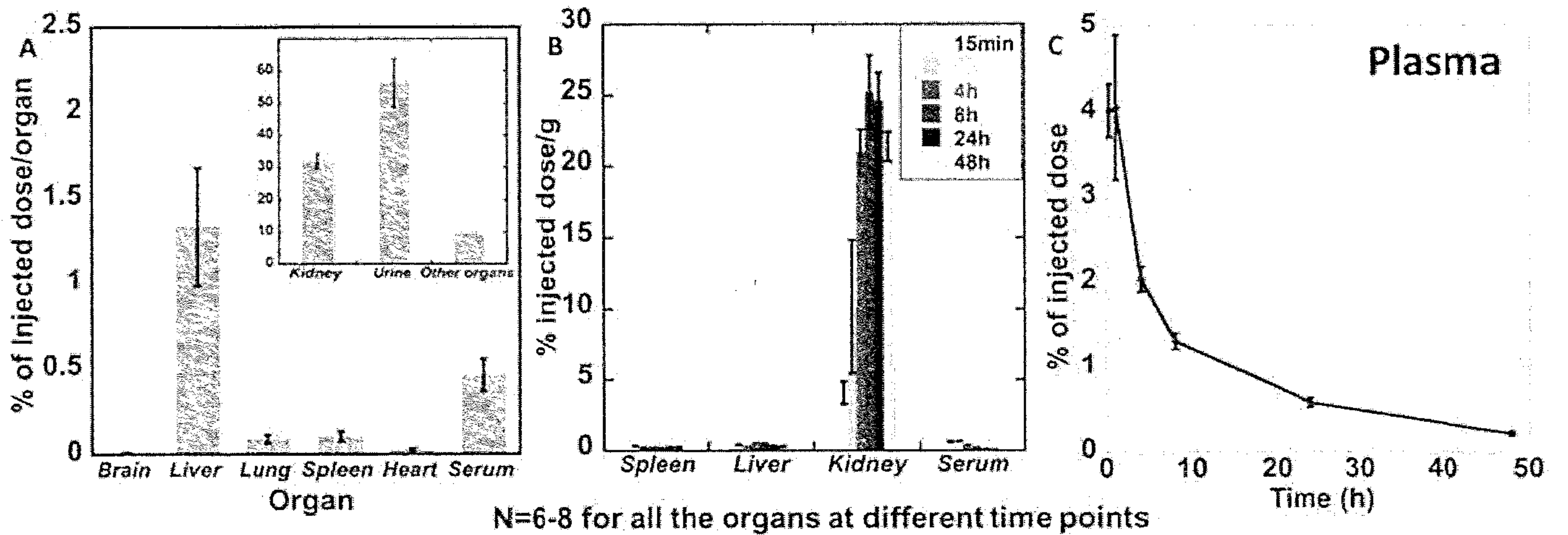


FIG. 5



FIGs. 6A-6C

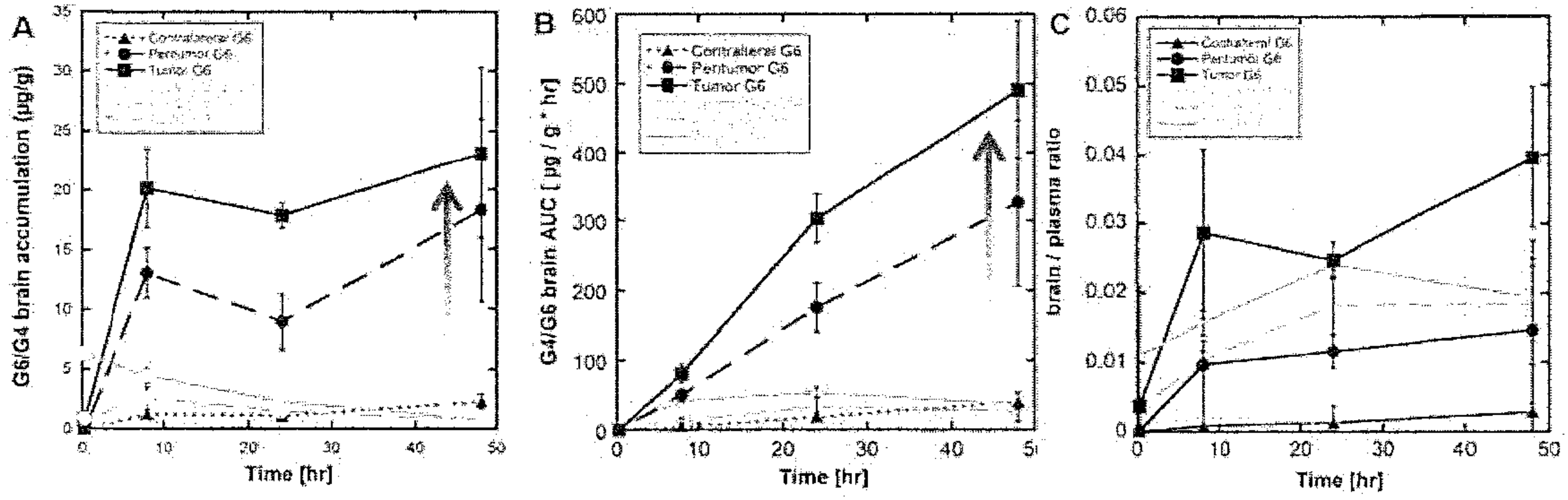
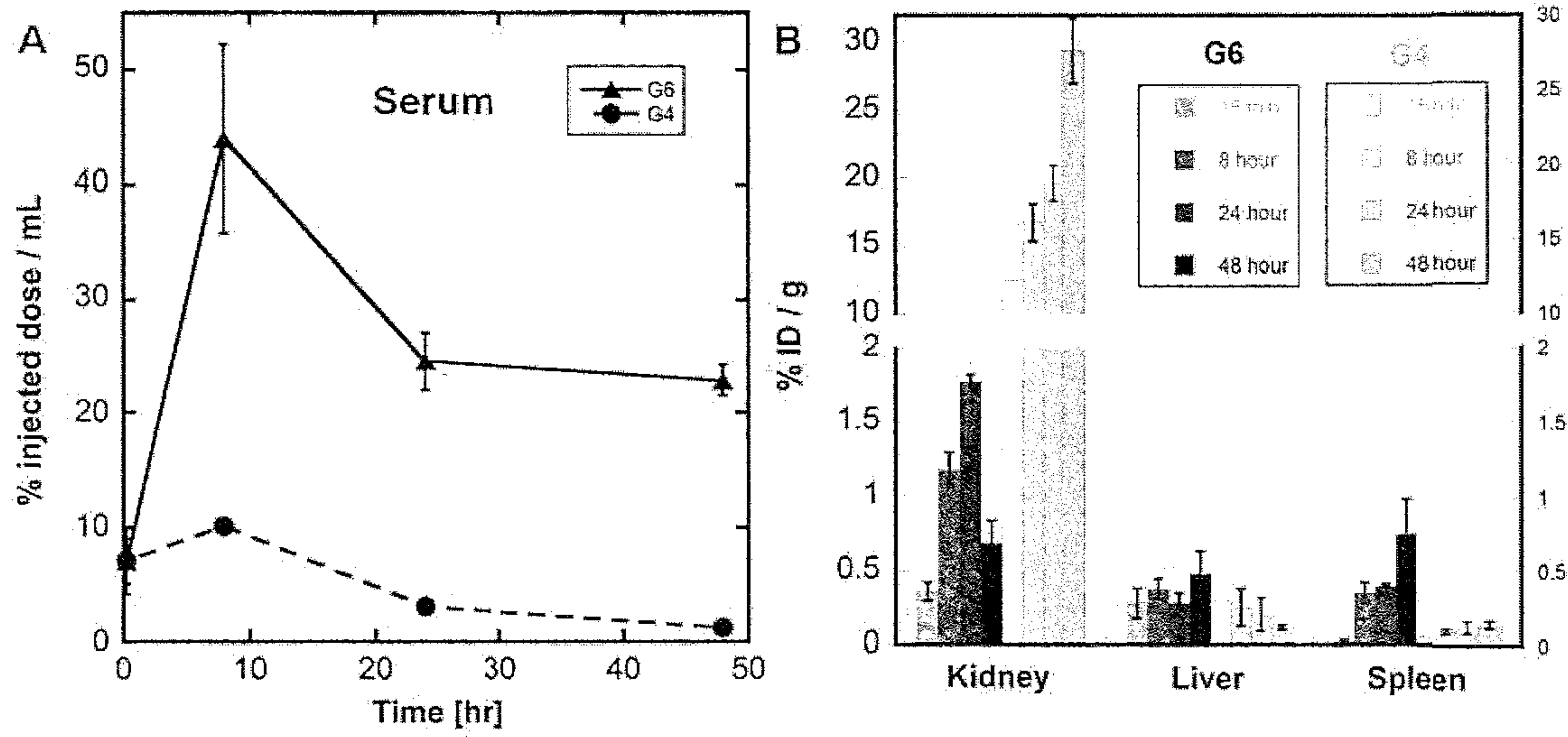


FIG. 7A-7C



FIGs. 8A- 8B