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Research Article

Targeted Boron Neutron Capture Therapy Using Polymalic Acid Derived Nano-Boron to Treat Glioblastoma

Patil R[1*], Sun T[2], Chepurna O[2], Khoobchandani M[1], Rudensky S[1], Holler E[3], Ljubimova JY[3], Black KL[2]

[1]Department of Basic Sciences and Neurosurgery, Division of Cancer Science, Loma Linda University, School of Medicine, Loma Linda, 11175 Campus St., Loma Linda, CA, 92350, USA

[2]Department of Neurosurgery, Cedars-Sinai Medical Center, Los Angeles, USA

[3]Terasaki Institute for Biomedical Innovation, Los Angeles, CA, USA

***Corresponding author:** Patil R, Department of Basic Sciences and Neurosurgery, Division of Cancer Science, Loma Linda University, School of Medicine, Loma Linda, 11175 Campus St., Loma Linda, CA, 92350, USA

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Abstract

Despite extensive efforts, glioblastoma multiforme (GBM), the most malignant brain cancer, continues to pose significant challenges to effective treatment, with limited progress in patient survival over the last three decades. This study addresses shortcomings of conventional therapies, particularly radiotherapy (RT), which faces limitations due to radio-resistance and toxic radiation doses. Boron neutron capture therapy (BNCT) is a promising alternative, delivering targeted radiation to tumor cells with minimal damage to healthy tissue. However, the key challenge lies in achieving sufficient boron uptake selectively in tumor cells. We have developed a novel nanomedicine-based approach, utilizing polymalic acid (PMLA) as a delivery vehicle, carrying multiple boron-10 molecules per nanoconjugate to increase the intracellular concentration of boron-10 for effective boron neutron capture therapy. Our novel nanodrug (Nano-Boron) incorporates isotopically enriched 4-boronophenylalanine (BPA) as a source of boron-10 and Angiopep-2 (AP2) peptide for blood-brain barrier penetration and tumor targeting. The PMLA platform allows for the attachment of a large quantity of boron-10, enhancing the intracellular boron concentration and, consequently, the efficacy of BNCT. This innovative approach holds the potential to address the unmet clinical need in GBM treatment and improve patient survival and quality of life.

Keywords: Glioblastoma Multiforme (GBM); Boron Neutron Capture Therapy (BNCT); Radiation Therapy; Angiopep-2 (AP2); Polymalic Acid (PMLA); 4-Boronphenylalanine (BPA).

Introduction

Despite substantial efforts and a wealth of recent data on glioma biology, there has been little advancement in enhancing patient survival over the last three decades [1-3]. According to the Central Brain Tumor Registry of the United States (CBTRUS), the incidence rate of malignant brain tumors is 7.08 per 100,000 in the US. In 2020, it was projected that around 83,830 new cases of both malignant and non-malignant tumors affecting the brain and other parts of the central nervous system would be diagnosed in the United States alone. Among these, approximately 24,970 cases were expected to be malignant, while the remaining 58,860 were non-malignant. Additionally, between 2013 and 2017, about 81,246 deaths were recorded due to malignant brain and other CNS tumors [4, 5]. Glioblastoma multiforme (GBM) stands out as the most aggressive form of brain cancer originating from astrocytes. While radiotherapy is a commonly employed treatment for GBM

patients, its efficacy often faces challenges due to the radioresistant nature of the aggressive GBM cells [1, 6].

Several formidable barriers are hampering the development of effective treatment strategies, including but not limited to 1) its highly infiltrative nature (growing many centimeters into surrounding viable/healthy brain); 2) its visual similarities to normal brain tissue (it is difficult to differentiate tumor from normal brain parenchyma for precise resection); 3) the tumor site (the location prevents surgical intervention without major neurological consequence); 4) the relative chemotherapeutic resistance/recurrence of brain tumors, and 5) the low therapeutic to toxic (α/β) ratio of radiation therapy (RT) in the brain. Each one of these issues is somewhat unique to brain tumors, and therefore a strategy to overcome each of them is critical to the development of effective treatments. RT has proven to be a key component of glioma treatment, providing ionizing radiation to tumor cells far beyond the primary tumor cavity. RT also plays a critical role in the treatment of unresectable tumors, brain metastases, and recurrent brain tumors. Yet, a major limitation of radiotherapy is that the dose of radiation required to achieve a therapeutic response (approximately 54-60 Gray (Gy)) is relatively close to the dose that is toxic to normal brain cells (approximately 70 Gy), which leads to necrosis and destruction of healthy brain tissues. This so-called α/β ratio is the primary limitation to radiotherapy [7]. Therefore, there is an unmet clinical need to develop a new modality that can overcome these problems and not only improve patient survival but also enhance the quality of life.

Boron neutron capture therapy (BNCT) represents a noninvasive treatment approach targeting locally invasive malignant tumors like glioma [8-12]. BNCT administers focused radiation precisely to tumor cells, minimizing harm to surrounding healthy tissue. Furthermore, it holds significant promise in addressing micro-metastases, which often elude detection through conventional medical imaging techniques [13]. BNCT is based on the principle of the nuclear interaction between the non-radioactive isotope boron-10 and low-energy (<10 keV) epithermal neutrons. In biological settings, boron-10 is loaded into target cells using agents such as L-p-boronophenylalanine (BPA) or sodium borocaptate (BSH). BPA has become the drug of choice due to its advantages over BSH and is preferred in many clinical trials, as it is non-toxic, and has no effect on its own [13-17]. When exposed to thermal neutrons, which cause little impact on tissue by themselves, the boron-10 nucleus captures a neutron and spontaneously undergoes a particle ejection reaction [$^{10}B(n,\alpha)\ ^{7}Li$] to generate high energy α-particles (^{4}He) and recoiling Lithium-7 (^{7}Li) particle, which are among the most strongly ionizing and destructive forms of radiation [18]. These particles with high linear energy transfer (LET) induce double-strand breaks (DSBs) in the DNA of cells, exhibiting approximately three times the relative biological effectiveness compared to photon or proton irradiation. Since the path length of an α-particle and recoiling Lithium-7 is approximately the same as the size of a single cell (5-9 μm), the cellular destruction effect is limited to the target cell, sparing all non-boron-10 containing cells [5]. In addition, the neutron cross-section of boron-10 is very high (3,837 barns) and many times greater than that of the other endogenous nuclei present in tissues such as hydrogen (0.33 barns), oxygen and carbon (<1.0 barn), and nitrogen (1.82 barn). Furthermore, the amount of radiation produced by neutron reactions with these elements is significantly less than the particle and recoiling nucleus in the case of boron [19]. An X-ray dose of ~1 Gy produces about 1000 single-stranded DNA breaks and about 50-100 DSBs in a typical mammalian cell and causes ~50% cell death. BNCT-based high LET α-particles cause high DSBs which is very difficult to repair by cellular machinery and may remain unrepaired leading to cell cycle arrest/death. Additionally, a dose equivalent effective for 60 Gy can be administered to boron-containing cells within a single hour, contrasting with the 5-6 weeks typically required for conventional radiotherapy.

While BCNT stands as a promising radiotherapy method for glioma and various other tumor types, a primary limiting factor has been achieving sufficient concentration of boron-10 in tumor cells using conventional drug delivery methods [20, 21]. Moreover, the boron-10 uptake needs to be specific to tumor cells alone (with a preferred ratio of 4:1 or better for tumor vs surrounding healthy brain), as non-specific uptake of boron-10 and subsequent exposure to a neutron beam would result in widespread destruction of healthy tissues, causing tremendous toxicity. This has become a major hurdle for BNCT to become a successful therapy. However, despite the availability of reactor-based neutron sources and the low ratios of boron-10 in tumors compared to healthy brain tissue using traditional small molecule-based methods, BNCT has emerged as a viable treatment option for newly diagnosed GBM, exhibiting effectiveness comparable to conventional RT alone [13, 22-24], with the advantage of a single intervention instead of multiple rounds of radiation and chemotherapy. Furthermore, for patients lacking unmethylated MGMT DNA repair gene, BNCT offers an edge over RT/Temozolomide (TMZ) [23]. BNCT has also been demonstrated to be effective in recurrent GBM, where current standard salvage treatments yield very dismal prognosis [14]. BNCT has also shown efficacy in recurrent GBM cases, particularly when standard salvage treatments offer poor prognoses. A recent study involving 34 brain tumor patients in critical, end-stage conditions demonstrated BNCT's ability to achieve an overall survival of 7.25 months without severe adverse events (grade ≥ 3) [25].

Presently, various accelerators designed for BNCT are undergoing assessment in clinical trials, [26, 27], awaiting findings. Despite the renewed momentum brought by the advancement of new

accelerators in BNCT [28], ensuring efficient delivery of boron-10 remains a significant hurdle that requires attention [26, 29]. A nanomedicine approach is uniquely poised to offer a solution to this unsolved problem. Nanomedicine in cancer treatment has been steadily on the rise [30, 31]. Nanodrugs offer several advantages over conventional small molecule drugs, such as improved solubility, increased plasma half-life, enhanced permeability and retention in tumors [32], active targeting [33-35], as well as decreased systemic toxicity and drug resistance [36]. The proposed nanodrug utilizes polymalic acid (PMLA) as its backbone carrier, which boasts natural derivation, biodegradability, non-toxicity, and non-immunogenicity [37]. PMLA features pendant carboxylic groups enabling the covalent attachment of various functional units. It has been extensively employed as a carrier for a range of therapeutic agents, antibodies, oligonucleotides, and imaging agents (both optical and MRI) capable of crossing the blood-brain barrier (BBB), showcasing promising clinical potential [33, 35, 37-39]. A compelling approach involves linking abundant boron-10 enriched molecules to a tumor-specific ligand to enhance payload through tumor-targeted delivery and uptake.

Our novel approach takes advantage of proven nanomedicine based on PMLA platform. Nanodrug (Nano-Boron) designed on a PMLA platform containing Angiopep-2 (AP2) peptide to cross BBB and target tumor cells [35, 39, 40] and isotopically enriched BPA as a source of boron-10 is proposed in this study. Figure 1 shows a schematic presentation of Nano-Boron based approach and BNCT principle. A major appeal of this strategy is that the PMLA provides numerous sites (600-800) for boron-10 attachment such that a single nanodrug containing a relatively small amount of targeting peptide (e.g. 1-2% of available sites in the PMLA backbone) could also contain large amounts of boron-10 (>300), markedly increasing the intracellular boron-10 concentration for highly effective BNCT outcome.

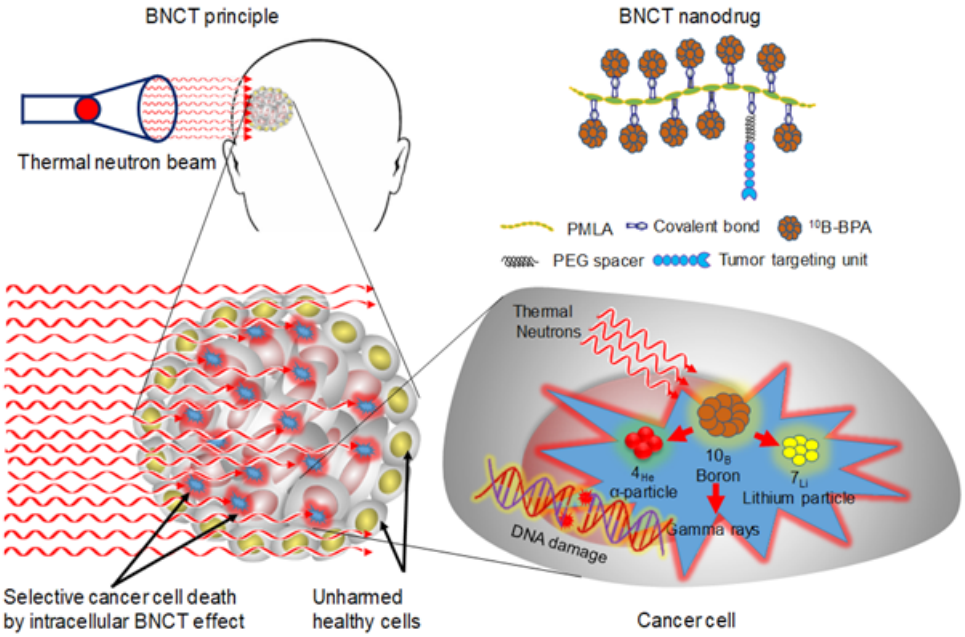

Figure 1: Schematic Presentation of Nano-Boron and BNCT principle.

Materials and Methods

Reagents

Polymalic acid (PMLA) with a molecular mass of 60 kDa and polydispersity of 1.2 (SEC-HPLC/polystyrene sulfonate standards) was isolated from the culture supernatant of Physarum polycephalum M3CVII, as previously described [41]. Rhodamine Red C2 maleimide was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Boron-10-enriched BPA, with >99% enrichment was purchased from Katchem spol., Prague, Check Republic. The peptide containing an added C-terminal cysteine Angiopep-2-cys (AP2) (TFFYGGSRGKRNNFKTEEY-C), was custom synthesized by AnaSpec (Fremont, CA, USA). All other chemicals and solvents of the highest purity were obtained from Millipore Sigma (Missouri, USA).

Synthesis of Preconjugate

N-Hydroxysuccinimide (NHS; 0.43 mmol) and N,N'-dicyclohexylcarbodiimide (DCC; 0.43 mmol) dissolved in 1.0 mL of dimethylformamide (DMF) were added consecutively to the solution of 50 mg of PMLA (0.43 mmol with regard to malyl units) dissolved in 1.0 mL of anhydrous acetone under vigorous stirring at RT. The reaction mixture was stirred at RT for 2 h to complete the activation of carboxyl groups. A solution of BPA, (44.8 mg, 50 mol % with regard to malyl units) was prepared in a mixture of 2.0 ml of DMF and 33 µL of trifluoracetic acid (TFA) and added to the reaction mixture, followed by the addition of 120 µL triethyl amine (TEA) in portions of 10 µL/5min. The reaction mixture was stirred at RT for the next 16 hours. 2-mercaptoethylamine (MEA) (0.04 mmol in DMF; 100 µL, 10 mol % with regard to malyl units) was added to the reaction mixture followed by an equivalent amount of TEA. The reaction mixture was stirred at RT for 45 min. 7.0 ml of phosphate buffer (100 mM sodium phosphate and 150 mM NaCl, pH 6.8) was added to the reaction mixture at RT and the reaction was further stirred for 1.0 h at RT to remove excess NHS. The final product was purified using PD-10 desalting columns, yielding a white floppy solid after lyophilization (isolated yield: 55-65%).

Conjugation of AP2 and Fluorescence Labeling

AP2 peptide was modified with terminal maleimide group to prepare AP2-PEG-MAL as described [39]. 10 mg/ml of AP2-PEG-MAL (in the buffer, 100 mM sodium phosphate pH 6.3) was dropwise added to a solution of preconjugate (4 mg/ml) at RT in the same buffer. The reaction was monitored by SEC-HPLC. After reaction completion (30 min), the pH of the reaction mixture was adjusted to 5.5 with 0.5 M citrate buffer, and 2 mg/ml MAL-functionalized ICG or Rh (dissolved in DMF at 2 mg/ml) was added. The reaction mixture was incubated at 4°C overnight. After reaction completion remaining free -SH groups were blocked with 3-pyridyldithiopropionate (PDP) [41]. The obtained nanodrugs were purified over PD-10 column in PBS, further passed through 0.2-micron pore filters, and later snap-frozen and stored at -20 °C until use. The synthesized agents are highly soluble in an aqueous buffer with the designed composition by chemical group analysis, protein assay, and UV quantitative photometry [41].

GBM Cell Line

Primary GBM cell line U87MG was obtained from ATCC (Manassas, VA, USA) and was cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum with a 1% mixture of penicillin (100 U/mL), streptomycin (100 µg/mL) at 37 °C with 5% CO_2. Cells were routinely checked for mycoplasma (a kit from Lonza, Bend, OR, USA) with negative results.

Tumor Inoculation

Athymic NCr-nu/nu female mice were obtained from Jackson Laboratory. Intracranial tumors were stereotactically implanted with U87MG GBM cells at a density of 5.0 x 10^4 per mouse into the right basal ganglia of the mouse brain. Throughout the study, all animals were monitored for neurological symptoms. All animal procedures adhered to ethical regulations for animal research and testing and were conducted under the approved protocol No. IACUC009043 by the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee (IACUC). To summarize the stereotactic intracranial tumor implantation procedure, a midline skin incision of 5-15 mm was made on the mouse's skull. Using a drill, a hole was created approximately 1-3 mm laterally or posteriorly and 1-3 mm rostrally from the Bregma. Subsequently, mice received a stereotactic intracranial injection of around 5.0 x 10^4 tumor cells into the right basal ganglia using a Hamilton syringe for over 1-2 minutes. The syringe was then slowly withdrawn over 5 minutes to prevent tissue contraction. The skull hole was promptly sealed with bone wax, and the incision was closed using a wound clip. After intra-cranial tumor implantation into the right basal ganglia of the mouse brain, all the animals were followed for neurological symptoms throughout the study.

Biodistribution

The ICG labeled Nano-Boron was injected intravenously (i.v.) via the tail vein at a BPA concentration of 50 mg/kg. Mice were euthanized by cervical dislocation. No perfusion was conducted during this process. The brain tumor was precisely removed under NIR image-guided resection using Synchronized near InfraRed Imaging System (SIRIS). Major organs (brain, lung, kidney, liver, heart, and spleen) were harvested and imaged. The boron-10 content in the resected tumor sample was measured by inductively cupelled plasma-mass spectroscopy (ICP-MS) and compared with surrounding healthy brain tissue. For the half-life of Nano-Boron drug, the whole blood was collected before (0 min) and

0.08, 0.5, 1.5, and 3 h after administration. 50 µl of blood was isolated at each timepoint, diluted (3X) in PBS EDTA mixture. Fluorescence intensity was recorded at 800 nm by Odyssey CLx (LI-CO Biotechnology, Lincoln, NE). A total of four mice (n = 4) were utilized for biodistribution study.

Fluorescent Staining for BBB Permeation

The rhodamine-labeled Nano-Boron was injected intravenously (i.v.) via the tail vein at a BPA concentration of 5.0 mg/kg. After 2h and 45 minutes, two lectins for labeling brain vascular endothelium were intravenously injected. This injection consisted of a combination of 75 µL of 1 mg/mL Lycopersicon Esculentum (Tomato) lectin DyLight 488 and 50 µL of 5 mg/mL Ricinus Communis Agglutinin I (RCA-I, RCA120) Fluorescein (both from Vector Laboratories, Burlingame, CA, USA). Mice were euthanized 3h after nanoboron injection. A total of three mice (n = 3) were utilized for imaging the permeation of the BBB and staining experiments. Brain tissue blocks, frozen after drug treatment, were sliced into sections measuring 7–10 µm using a Leica CM 3050S cryostat (Leica Microsystems, Buffalo Grove, IL, USA). After slicing the tissue sections, it was air-dried at room temperature (RT), fixed with ice-cold acetone for 10 minutes, rinsed thrice with PBS, and then mounted. Imaging was conducted using a Leica DM6000B microscope (Leica Microsystems, Buffalo Grove, IL, USA). Direct fluorescence immunohistochemistry was employed utilizing labeled lectins.

Statistical Analysis

Statistical analysis of boron-10 quantification in each cohort was carried out using Prism 8 program (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

Results

Synthesis of Nano-Boron

A novel synthetic method to covalently attach BPA to PMLA and to a backbone via a stable amide linkage was developed. As represented in the synthetic scheme in Figure 2, intermediates (preconjugate and AP2-MAL) were prepared separately and conjugated to form a Nano-Boron. Ensuring high reproducibility and precision in synthesizing PMLA-based Nano-Boron is crucial and poses a challenge for multifunctional nanomedicines, particularly in successful combination therapy against cancer. We have attained reproducible synthesis of diverse nanodrugs and intermediates, meticulously controlling the conjugation of each component. The synthesis of the PMLA nanoconjugate typically involves a two-step process. PMLA purified from the culture supernatant of Physarum Polycephalum (>97% purity, Mw 60 kDa, polydispersity P = 1.2) was used for all Nano-Boron. First, PMLA was activated at –COOH by the standard NHS/DCC method followed by sequential amination with BPA and 2-mercaptoethyl-1-amine (DMF/triethylamine) to form a preconjugate. Further, it is lyophilized for long-term storage and conveniently used to prepare various functional nanoconjugates. The preconjugate was purified using a PD-10 desalting columns, further, it was lyophilized, and stored at -20 °C. Nano-Boron drug contained 50% loading of BPA, along with 2% loading of AP2 targeting molecule and 1% loading of fluorescent dyes such as Rhodamine or indocyanine green.

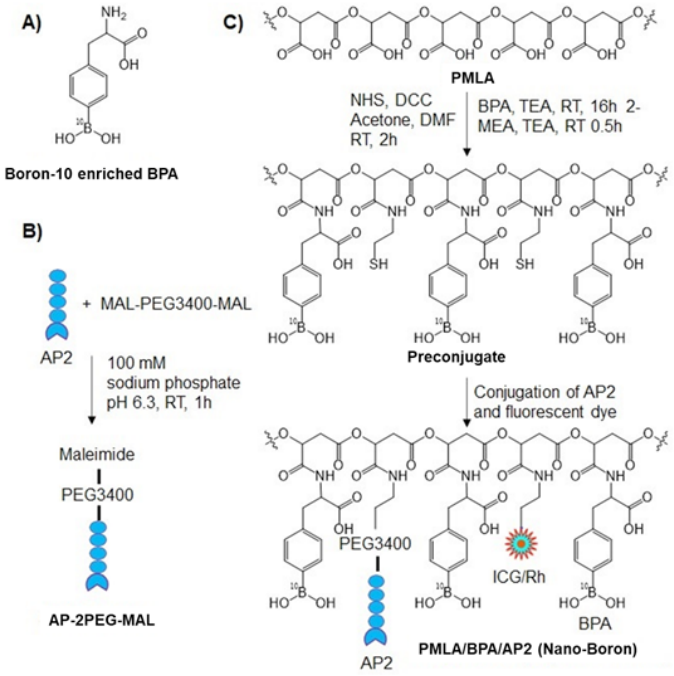

Figure 2: Synthesis of intermediates and Nano-Boron.

Physicochemical Characterization

Detailed physicochemical characterization of nanodrug was performed using state-of-the-art techniques such as high-performance liquid chromatography (HPLC), dynamic light scattering (DLS), UV-vis spectrophotometry along with basic physicochemical and analytical assays (wet chemistry) to quantify the amount of each component on the nanodrug. These techniques/assays are well established in our lab and have been routinely used for nanodrug characterization [33-35, 37, 41, 42]. ICP-MS has been widely used for boron-10 analysis [43]. The amount of boron-10 in nanodrugs was quantified in tissue samples at Element, Santa Fe Springs, CA USA. Detailed physicochemical characterization is summarized in Table 1.

Boron-10 Nanodrugs	Abbreviation	Size[a] (nm)	Zeta Potential[b] (eV)
PMLA/BPA(50%)/AP2(2%)/ICG(0.5%)[c]	PMLA/BPA/AP2/ICG	6.6 ±1.	-8.3 ±1.1
PMLA/BPA(50%)/AP2(2%)/Rh(0.5%)	PMLA/BPA/AP2/Rh	ND[d]	-6.1 ±0.4

Table 1: Physicochemical characterization of first-generation Nano-Boron variants.

[a]Measured by volume distribution at 25 °C measured in PBS at a concentration of 2 mg/ml, calculated from DLS data by the Malvern Zetasizer software (Malvern Instruments, Malvern, UK). [b]Zeta potential at 25 °C in aqueous solution of 10mM NaCl at 150 mV. [c]Composition of nanodrug; percentage refers to the total number (100%) of pendant carboxyl groups in unsubstituted PMLA. [d]Not determined due to interference of rhodamine fluorescence with the ZS90 detector.

BBB Delivery of Nano-Boron in Mouse Model

Previous studies have demonstrated that the conjugation of PMLA to BBB delivery vectors containing the AP2 peptide results in high brain permeability [25, 44]. Mouse models of orthotopic brain cancer were prepared according to the established method with an IACUC approved protocol. Stereotactic intracranial injection of approximately 50,000 of U87MG tumor cells to the right basal ganglia was performed. This glioma model is a frequently used tumor model to study GBM, is highly invasive with many characteristics of GBM phenotypes, and has been well established in our lab with median survival ranging from 30-35 days for U87MG [35, 38, 45].

Pharmacokinetic and Biodistribution of Nano-Boron in GBM Models

ICG labeled Nano-Boron was i.v. injected via the tail vein. Mice were euthanized by cervical dislocation, 3h after the Nano-Boron administration. The brain tumor was precisely removed under NIR image-guided resection using SIRIS. It is designed to detect picomolar concentration of ICG and has been successfully used in our preliminary results for ICG labeled Nano-Boron drug detection in real-time for precise tumor resection in mouse models of glioma [31]. Blood was drawn at several time points and the biological half-life life of Nano-Boron was found to be 86.14 minutes (Figure 3B). Major organs (brain, lung, kidney, liver, heart, and spleen) were harvested and imaged (Figure 3 A). Nano-Boron was selectively accumulated in the tumor vs healthy brain at a ratio of 16.2:1 and BPA concentration in the tumor was found to be around 62.7 ± 18.2 ppm while 3.8 ± 1.0 ppm was detected in the healthy brain (Figure 3C).

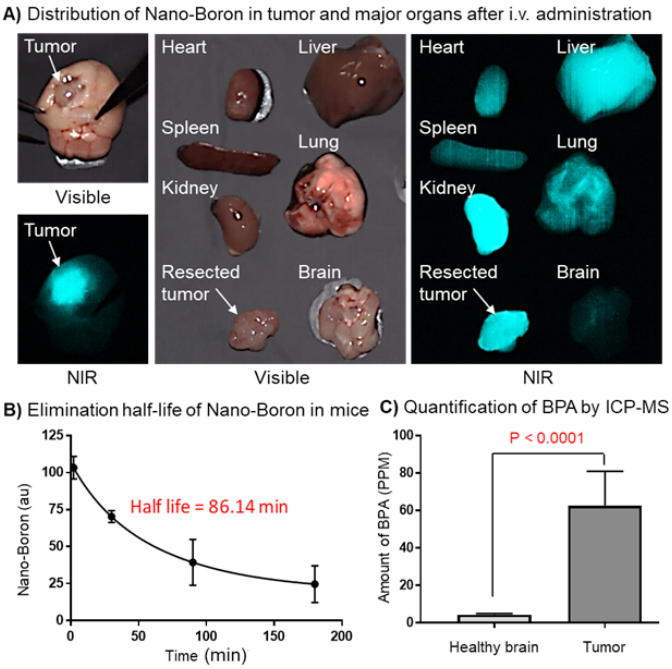

Figure 3: Pharmacokinetic and biodistribution studies of Nano-Boron in GBM mouse model. The xenograft mouse model of U87MG was used for this study. A) Visual inspection of ICG labeled Nano-Boron in various vital organs using SIRIS imaging system. Nano-Boron shows selective accumulation in tumor vs surrounding healthy brain. B) The elimination half-life of the Nano-Boron drug was found to be 86.14 min. C) Quantification of BPA in tumor vs surrounding healthy brain. Nano-Boron drug is selectively accumulated in tumors at a ratio of 1:16.2 (healthy brain: tumor). 4 mice were used to study pharmacokinetics and biodistribution.

Intracellular Delivery of Nano-Boron Drug across BBB

Rh labeled Nano-Boron was i.v. injected via the tail vein at a BPA dose of 5.0 mg/kg. 15 min before euthanasia, mice were injected with fluorescein-labeled lectin dyes (tomato and ricin, to stain the normal and tumor blood vessels respectively). 3 h after the Nano-

Boron injection, mice were euthanized, and their brain was sectioned and evaluated under the fluorescent microscope. Nano-Boron was preferentially located in the tumor area, evident by intense fluorescence intensity only in the tumor area (Figure 4, top panel). Higher magnification (Figure 4, bottom panel) revealed high intracellular fluorescence intensity (white arrows). Three mice were used in this cohort study to image the tissue samples, and representative images are shown in Figure 4.

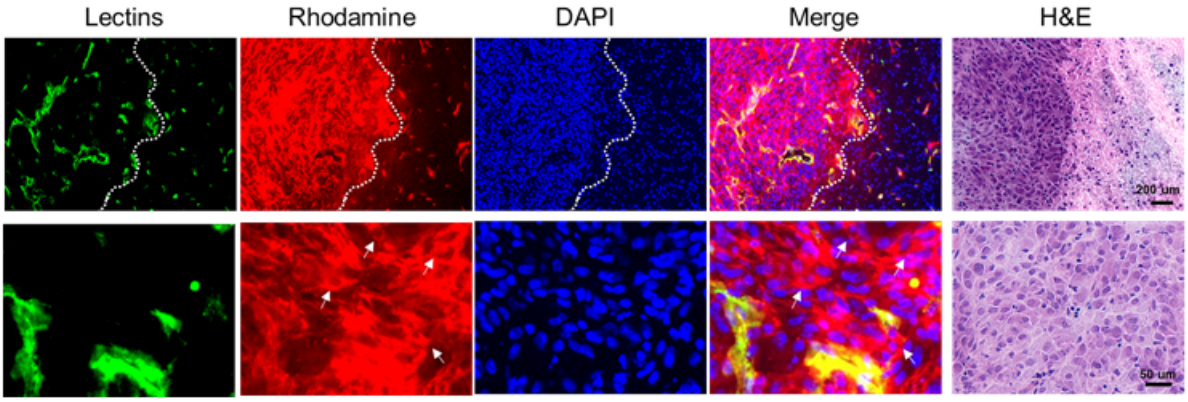

Figure 4: BBB delivery of Nano-Boron in a mouse model of human U87MG glioma after i.v. injection. The white dotted line represents the tumor border. Green channel: fluorescein-labeled lectin dyes (tomato and ricin to stain the normal and tumor blood vessels respectively). Red channel: Rh labeled nanodrug shows preferential accumulation in tumors with strong intracellular intensity (white arrows). Blue channel: DAPI for nuclei. H&E shows relatively similar areas of the section used for IHC staining.

Discussion

The integration of nanotechnology into cancer therapy holds immense promise for revolutionizing clinical practice. The utilization of nanodrugs holds several distinct advantages over conventional cancer therapies [46-48]. Firstly, nanomedicine allows for the customization of drug delivery systems tailored to specific tumor types, thereby enhancing therapeutic efficacy while minimizing systemic toxicity [47]. Moreover, nanodrugs exhibit prolonged circulation times in the bloodstream, leading to enhanced bioavailability and improved pharmacokinetics [49]. This not only enhances drug accumulation at tumor sites but also facilitates sustained release kinetics, optimizing the therapeutic window [47, 49]. The present study underscores the transformative potential of nanodrug formulations in addressing the challenges associated with conventional cancer therapies. In our research, Nano-Boron for BNCT is being developed using several crucial components, namely Poly (malic acid) (PMLA), boron-10 enriched BPA drug, tumor-targeting peptide (AP2), and fluorescent dye Indocyanine green (ICG). Each component serves a specific purpose in enhancing the efficacy and safety of the Nano-Boron.

PMLA is biodegradable and can be broken down into non-toxic byproducts by natural processes within the body. This property is advantageous for biomedical applications as it reduces the risk of long-term accumulation and toxicity. In addition, PMLA can be easily functionalized or modified to incorporate targeting ligands, imaging agents, or therapeutic payloads, allowing for the development of multifunctional Nano-Boron tailored for specific applications [37]. Furthermore, PMLA nanoconjugates can improve the solubility and stability of hydrophobic drugs, thereby enhancing their bioavailability and therapeutic efficacy [39]. PMLA-based nanoconjugates can be designed to achieve sustained and controlled release of encapsulated drugs, providing persistent therapeutic effects, and reducing the frequency of dosing [36]. As demonstrated in our previous studies, PMLA-AP2 nanoconjugates exhibit selective binding affinity towards glioma cells while showing minimal binding to normal brain tissues [39]. This selective targeting enhances the specificity of drug delivery and reduces off-target effects. Moreover, PMLA-AP2 nanoconjugates have been shown to efficiently cross the BBB, enabling the delivery of therapeutic agents to the central nervous system for the treatment of brain tumors and neurological disorders [39]. PMLA nanoconjugates can be utilized for imaging applications, such as fluorescence imaging, enabling non-invasive visualization and monitoring of disease progression or therapeutic response, similar to our present study. These properties collectively make PMLA nanoconjugates a promising platform for targeted drug delivery of boron-10 molecules for improved BNCT.

Additionally, our approach involves active targeting using a synthetic AP2 peptide (TFFYGGSRGKRNNFKTEEY), as identified by Demeule et al [50], which exhibits efficient crossing of the BBB [39]. The transport of AP2 reaches saturation at higher concentrations and is hindered by other ligands of low-density

lipoprotein receptor-related protein-1 (LRP-1) [41], affirming the transcytosis of AP2 across the BBB. AP2 has been effectively employed for BBB transport by various research groups [41, 42, 44, 45, 51] including our own [39] to shuttle a variety of nanodrugs based on different carriers including PMLA-LLL across the BBB. Furthermore, BPA, an amino acid analog closely resembling L-phenylalanine, exhibits favorable accumulation in gliomas through the L-type amino acid transporter-1 (LAT-1), which is upregulated in the BBB and gliomas [52] and is highly prevalent in brain metastasis [53]. The study underscores the potential of nanomedicine in cancer treatment, emphasizing the advantages of nanodrugs over conventional therapies. These include improved solubility, increased plasma half-life (Figure 3), enhanced tumor targeting, and reduced systemic toxicity [39, 54]. The use of PMLA as a backbone for Nano-Boron drugs adds the benefits of biodegradability and non-toxicity [39, 54].

In addition to targeted delivery, in the present research, we are focusing on developing a targeted nanodrug with enriched boron-10 loaded to treat GBM using BNCT. One of the major obstacles to effective BNCT lies in achieving optimal boron uptake within tumor cells. To address this challenge, we've devised an innovative nanomedicine-based approach. This strategy leverages the unique properties of PMLA as a delivery vehicle, meticulously engineered to transport multiple boron-10 molecules [39, 55]. By significantly enhancing the intracellular concentration of boron-10, our method aims to unlock the full therapeutic potential of BNCT with unprecedented precision and efficacy. We synthesized varied loading of BPA to PMLA (20-70 % with regards to PMLAs pendant carboxylates). It is noted that a high loading of BPA (>50%) caused crowding of BPA on PMLA backbone, leading to aggregation of preconjugates. Hence, we kept the BPA conjugation to an optimum loading of 50% with regard to the pendent carboxylate groups.

Our Nanodrugs exhibit optimum circulation times in the bloodstream due to their nano-sized dimensions, which mitigate rapid clearance by the reticuloendothelial system. This extended plasma half-life allows for sustained drug release kinetics, maintaining therapeutic concentrations within the bloodstream over an extended period (Figure 3 B, C). Consequently, this nanodrug also offer enhanced pharmacokinetic profiles (Figure 3) compared to conventional therapies, enabling less frequent dosing regimens, and reducing the risk of dose-related toxicities [39]. The size of Nano-Boron is around 6.6 nm, which is lower than the renal clearance size of 8.0 nm [56, 57], allowing it to clear relatively faster, whereas due to the active targeting via AP2 it gets accumulated in the tumor area. This unique ability of nanodrug makes an ideal candidate for boron-10 delivery.

A hallmark feature of nanodrug formulations is their ability to selectively target tumor tissues while sparing healthy surrounding cells. This targeted approach utilizes the active targeting using AP2 peptide to facilitate targeted delivery to glioblastoma cells. Our findings underscore the importance of incorporating ligand-specific targeting strategies to enhance therapeutic efficacy. By exploiting the aberrant physiology of tumor vasculature and the overexpression of certain receptors on cancer cells, nanodrugs can achieve preferential accumulation within tumor microenvironments, maximizing drug concentrations at the site of action. In addition to leveraging the targeting AP2 peptide for BBB crossing, the conjugation of multiple molecules of BPA to PMLA presents an opportunity to enhance nanodrugs transport across the BBB and augment tumor accumulation through multitargeting of BPA via LAT-1 transporters. We have utilized similar approach using peptide targeting in our earlier study [54].

Finally, the PMLA-based nanodrugs have been subjected to extensive safety and toxicity evaluations, demonstrating remarkable biocompatibility even at elevated concentrations [37, 55]. This robust safety profile positions them as promising candidates for further advancement in clinical nanodrugs. In clinical settings, the administration of BPA in humans via intravenous infusion typically ranges from 200-600 mg/kg/h over a 2-hour period, with a continuous infusion at 100 mg/kg/h during neutron irradiation, resulting in a cumulative BPA dose exceeding 500 mg/kg [18]. Notably, BPA exhibits limited aqueous solubility [58], but its solubility can be enhanced through complexation with fructose, a method utilized in many clinical studies [29, 59]. In contrast, all variants of our Nano-Boron formulations demonstrate exceptional water solubility and are administered at approximately one-tenth of the BPA dose (50 mg/kg). Nonetheless, our nanodrugs accumulated in brain tumors with a very high selectivity over healthy brain tissue. This would greatly reduce any offsite targeting during neutron irradiation. In addition, longer retention in tumors via active targeting allows a relatively larger window for neutron irradiation. Nanoconjugates, especially those employing PMLA as a base, show potential for precise drug delivery and therapy across diverse diseases. Active targeting methods and thorough safety assessments reinforce the feasibility of nanodrugs in clinical settings, especially in BNCT for brain tumors.

Conclusions

The success of BNCT hinges on two main factors. A reliable source of in-hospital based low energy neutron source and selective drug delivery to malignant cells in high concentrations. While the recent development in accelerator-based neutron sources has given a new impetus to the BNCT, drug delivery remains a major limitation. Our research proposes a Nano-Boron as a potential solution, offering a targeted and efficient delivery of boron-10 to GBM. The attachment of isotopically enriched boronophenylalanine (BPA) to the PMLA backbone, along with the inclusion of AP2 peptide

for blood-brain barrier penetration, positions the Nano-Boron as a promising candidate for overcoming the limitations of current therapies. Moreover, our results suggest that Nano-Boron might facilitate improved and localized intracellular delivery of boron-10, thereby presenting BNCT as a feasible and highly effective treatment approach for GBM and potentially other malignancies. The potential clinical impact is underscored by the nanodrug's solubility, low toxicity, and ability to deliver therapeutic doses at lower concentrations compared to current clinical practices.

Disclosure

Author Contributions: Conceptualization, R.P.; methodology, R.P.; validation, T.S., O.C. and S.R.; writing—original draft preparation, O.C. and M.K.; writing—review and editing, R.P., J.Y.L., E.H. and K.L.B; project administration, R.P.; funding acquisition, R.P.

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