

BIOLOGY CONTRIBUTION

L-DOPA PRELOADING INCREASES THE UPTAKE OF BOROPHENYLALANINE IN C6 GLIOMA RAT MODEL: A NEW STRATEGY TO IMPROVE BNCT EFFICACY

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Purpose: Boron neutron capture therapy (BNCT) is a radiotherapeutic modality based on $^{10}\text{B}(n,\alpha)^7\text{Li}$ reaction, for the treatment of malignant gliomas. One of the main limitations for BNCT effectiveness is the insufficient intake of ^{10}B nuclei in the tumor cells. This work was aimed at investigating the use of L-DOPA as a putative enhancer for ^{10}B -drug 4-dihydroxy-borylphenylalanine (BPA) uptake in the C6-glioma model. The investigation was first performed *in vitro* and then extended to the animal model.

Methods and Materials: BPA accumulation in C6-glioma cells was assessed using radiowave dielectric spectroscopy, with and without L-DOPA preloading. Two L-DOPA incubation times (2 and 4 hours) were investigated, and the corresponding effects on BPA accumulation were quantified. C6-glioma cells were also implanted in the brain of 32 rats, and tumor growth was monitored by magnetic resonance imaging. Rats were assigned to two experimental branches: (1) BPA administration; (2) BPA administration after pretreatment with L-DOPA. All animals were sacrificed, and assessments of BPA concentrations in tumor tissue, normal brain, and blood samples were performed using high-performance liquid chromatography.

Results: L-DOPA preloading induced a massive increase of BPA concentration in C6-glioma cells only after a 4-hour incubation. In the animal model, L-DOPA pretreatment produced a significantly higher accumulation of BPA in tumor tissue but not in normal brain and blood samples.

Conclusions: This study suggests the potential use of L-DOPA as enhancer for BPA accumulation in malignant gliomas eligible for BNCT. L-DOPA preloading effect is discussed in terms of membrane transport mechanisms. © 2008 Elsevier Inc.

BNCT, C6-glioma, rat brain, L-DOPA, BPA, HPLC, MRI, Dielectric Spectroscopy.

INTRODUCTION

Malignant gliomas are the most common primary intracranial neoplasms in humans, accounting for about 78% of all malignancies of the central nervous system (1). More than 80% of these tumors are considered high-grade (Grades 3 and 4) according to the current World Health Organization criteria (2). Anaplastic astrocytomas (Grade 3) and glioblastomas (Grade 4) are typically associated with a severe prognosis, with a median overall survival of 1–5 years (2). Despite advances in microsurgical techniques, radiotherapy, and chemotherapy, there has been little improvement in the clinical outcome of patients suffering from these kinds of tumors.

Boron Neutron Capture Therapy (BNCT) (3–6) is a technique based on a targeted radiation approach, which represents an alternative adjuvant therapy for malignant gliomas. It has already been used in patients with various types of brain malignancies, including glioblastomas (7–12), anaplastic meningiomas (13), cerebral melanoma metastases (14), or tumors recurrences in inoperable anatomic locations (15, 16). In this context, a few Phase I (7, 8, 10, 11) and Phase II studies (8, 10) have consistently demonstrated no severe effects of BNCT-related toxicity, and some preliminary evidence of therapeutic effectiveness (7, 9, 12) has been presented. From a technical point of view, BNCT is based on the

intratumoral delivery of a stable boron isotope (^{10}B nucleus) featuring high cross-section for thermal neutrons capture. In the presence of an adequate amount of ^{10}B within the tumor cells (approximate minimal effective dose: 20–35 $\mu\text{g/g}$), irradiation with low-energy (thermal or epithermal) neutron particles ($^{10}\text{B} + n_{\text{th}} \rightarrow \alpha + ^7\text{Li} + 2.79 \text{ MeV}$) induces a short-range nuclear reaction. As a consequence, heavy charged particles (^7Li and α particles) characterized by high linear energy transfer (LET) produce a disruptive effect within a spatial range of 5–10 μm . These characteristics ensure a well-localized effect of BNCT, which is virtually confined to the cells containing a critical amount of ^{10}B . A more selective and higher accumulation of ^{10}B in the tumor cells will result in a more effective and less toxic BNCT. As mentioned earlier, some clinical trials have reported encouraging results on the use of BNCT in patients with glioblastomas. However, the clinical outcome is still considered largely unsatisfactory. It is general opinion that one of the major limitations for BNCT effectiveness is the insufficient incorporation of ^{10}B into the tumor cells, even considering the most advanced methods of ^{10}B administration (17–20). An additional limitation of BNCT is the relatively low specificity of ^{10}B uptake in tumor cells as compared with the surrounding normal tissues.

Thus far, two ^{10}B carriers have been used for clinical purposes: the mercapto-undecahydrododecaborate, $\text{Na}_2\text{B}_{12}\text{H}_{11}\text{SH}$ (BSH) (21) and the *p*-dihydroxyboryl-phenylalanine $\text{C}_9\text{H}_{12}\text{BNO}_4$ (BPA) (22). A large body of literature has demonstrated a higher effectiveness of BPA compared with BSH in the BNCT of brain malignancies (17, 23, 24). This might be explained by a different microdistribution and tumor incorporation of the two compounds (25–28). BPA is believed to pass through the blood brain barrier (BBB) and cell membranes, and it is found at higher concentrations in tumor cells (29). The details of the uptake mechanisms for BPA into tumor cells are still not completely understood. There is evidence that such an uptake is supported by a carrier-mediated transport rather than by passive diffusion. Some authors have demonstrated that the administration of L-tyrosine increases the intracellular accumulation of BPA in mouse melanoma cells (30). This effect has also been replicated in 9L rat gliosarcoma cells (31). Moreover, BPA accumulation in 9L rat gliosarcoma cells was enhanced by either pretreatment with molecules targeted by L or A aminoacid transport systems. These findings suggest that such transporters work with a substrate-coupled antiport (exchange) mechanism, which is enhanced by preloading of specific aminoacids. L-DOPA is a well-known molecule with a chemical structure similar to those of L-tyrosine and BPA. A previous *in vitro* study on 9L rat gliosarcoma cells has demonstrated that L-DOPA preloading improves several times the intracellular accumulation of BPA (31). Conversely, a simultaneous incubation of 9L gliosarcoma cells with L-DOPA and BPA causes a decrease of intracellular BPA accumulation. These observations are particularly interesting not only for biological speculation but also for their impact on potential clinical applications.

In fact, the large experience accumulated in the use of L-DOPA (at different doses) as a treatment for Parkinson disease (32, 33) might allow its employment as a potential enhancer of BPA accumulation in BNCT clinical trials for malignant gliomas.

These considerations prompted the current work, which focused on investigating the potential role of L-DOPA in BNCT. We adopted for our experiments the C6 glioma cell line, which has been widely used to evaluate *in vitro* the effects of novel therapies and to produce animal models based on tumor-cell implantation. Specific aims of this study were as follows: (1) to replicate in C6 glioma cells the findings previously described in 9L gliosarcoma cells (31) by demonstrating a significant increase of BPA intracellular accumulation due to L-DOPA preloading (Experiment 1); (2) to assess *in vivo*, using the C6 glioma cell rat model, the effect of L-DOPA preloading on BPA accumulation in tumors as compared to normal brain tissue (Experiment 2). Experiment 1 was conducted using radiowave dielectric spectroscopy, which allows passive electric parameters of cell membrane to be measured (34)—namely, the permittivity (ϵ_s) and the electrical conductivity (σ_s) of the cell membrane and the electrical conductivity (σ_p) of the intracellular medium (cytosol). Changes of σ_p have been shown as proportional to variations in intracellular BPA content (27). In Experiment 2, BPA quantification in animal tissues was performed using high performance liquid chromatography (HPLC). As previously shown, this technique is a reliable method for analysis of BPA incorporation in several biological tissues, including cerebral samples (35).

METHODS AND MATERIALS

Experiment 1

This experiment was performed by radiowave dielectric spectroscopy, adopting the same procedure previously employed to assess the intracellular accumulation of BPA and BSH in C6-glioma cells (27). A detailed description about cell cultures and radiowave spectroscopy measurements may be found elsewhere (27, 34). Briefly, C6 glioma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 $\mu\text{g/mL}$ of gentamicin at 37°C under an atmosphere of 5% CO_2 in air. Each sample included about 1.6×10^8 cells. The first step was to generate a reference sample to assess the basic characteristics of permittivity and electrical conductivity σ_s of the C6 glioma cell membranes. Equivalent samples were then produced to investigate changes of the electrical conductivity σ_p under five experimental conditions: (1) cells incubated with addition of 2 mmol BPA (Condition 1); (2) cells incubated with addition of 50 $\mu\text{g/mL}$ of L-DOPA for 2 hours (Condition 2); (3) cells incubated with addition of 50 $\mu\text{g/mL}$ of L-DOPA for 4 hours (Condition 3); (4) cells incubated with addition of 2 mmol BPA after a 2-hour preincubation with 50 $\mu\text{g/mL}$ L-DOPA (Condition 4); (5) cells incubated with addition of 2 mmol BPA after a 4-hour preincubation with 50 $\mu\text{g/mL}$ L-DOPA (Condition 5). Before any dielectric measurement, each sample was washed twice with fresh serum-free culture medium. Dielectric measurements were carried out at 37°C, by evaluating permittivity $\epsilon(\omega)$ and conductivity $\sigma(\omega)$ of whole cell suspensions in a frequency range from 1 kHz to 1 GHz. The conductivity value of the cytosol (σ_p) was obtained by fitting, within the effective medium theory

approximation (36), an appropriate mixture equation based on a single-shell model to the experimental $\varepsilon(\omega)$ and $\sigma(\omega)$ spectra. This parameter, which probes the whole ionic content within the cytosol, is influenced by the intracellular concentration of BPA, L-DOPA, or both. For each experiment or condition, conductivity cytosol changes were reported as percentage differences $(\sigma_p^{\text{test}} - \sigma_p^{\text{ref}}) / \sigma_p^{\text{ref}}$ between values measured in the test- (σ_p^{test}) and in the reference-sample (σ_p^{ref}), respectively.

Experiment 2

Thirty-two male Wistar rats weighing 300–350 g were used for the current experiment. All procedures related to animal care were performed in accordance with Decree 116/92, which represents the Italian enforcement of the European Directive 86/609/EEC. In the first step of Experiment 2, each rat, intraperitoneally anesthetized with chloralium hydrate (1.5 mg/Kg), underwent brain implantation of partially deantigenized C6 glioma cells. The head of each rat was held into a stereotaxic frame (produced by Stoelting, IL), and a burr hole (3 mm) was made using a hand drill at the right calvarium (stereotaxic coordinates: 2 mm anterior to the right coronal suture and 5 mm to the right of the midline). Culture medium (10 μ L) containing 10^6 cells was then inoculated at a depth of 4 mm from the dural surface using a Hamilton syringe with a 26-gauge needle. The needle was slowly removed 2 min after the injection. The burr hole was covered with candle wax, and the scalp was sutured. No complications were encountered during the surgical procedure. Brain MRI monitoring was used to assess tumor implantation and growth in each rat. Five days after surgical implantation of C6 glioma cells, each rat underwent a first MRI scan (baseline) using a system operating at 7T (Bruker Biospec 70/15, Ettlingen, Germany), equipped with a 40-mm-diameter ^1H surface coil. Rats were reanesthetized with chloralium hydrate (1.5 mg/Kg) and positioned on a homemade bed with the head secured to the surface coil. T1-weighted (repetition time/echo time = 1200/12.9 ms) and T2-weighted (repetition time/echo time = 2500/40 ms) Spin Echo sequences were acquired with a 128×128 matrix size, a 40-mm field of view, and a $312 \times 312 \mu\text{m}$ of in plane image resolution. In both sequences, four contiguous 1.5-mm-thick slices were collected to cover the entire brain. Using the same acquisition protocol, all rats underwent serial MRI assessments (once every 4 days) for longitudinal monitoring of tumor growth. When the tumor size reached a minimum diameter of 2.0 mm on T2-weighted images, rats were randomly assigned to one of the two experimental branches: (1) BPA administration with L-DOPA pretreatment or (2) BPA administration without L-DOPA pretreatment (control group). Animals belonging to the first branch ($n = 16$) received 50 mg/Kg L-DOPA intraperitoneally 24 hours before BPA administration. This large dose of L-DOPA and the long time gap were chosen to obtain a prolonged L-DOPA availability than that observed at lower doses (37), with negligible competition effects for BBB transport between L-DOPA and its metabolites and for BPA. Indeed, a 15-hour half-life has been estimated for the 3-MT metabolite in rats treated with L-DOPA (38). Each rat was then anesthetized again as described earlier, and its right internal carotid was catheterized using a 0.7-mm cannula. This access was immediately used for the infusion of 300 mg/Kg BPA-fructose complex, administered over 30 min by a constant flow pump (Harvard Apparatus, Cambridge, MA). Animals belonging to the control branch ($n = 16$), underwent the same procedure (right internal carotid incannulation, infusion of BPA-fructose complex) with no preadministration of L-DOPA. Seven animals died from complications during the carotid infusion of BPA. The 25 survivors (15 from branch 1 and 10 from branch 2)

were sacrificed by craniotomy 150 min after BPA infusion because at this time the higher tumor-to-ipsilateral brain BPA ratios are expected (39). Tumor tissue, normal brain (obtained from both hemispheres, ipsi- and contra-lateral to the tumor implantation), and blood samples were collected for HPLC analysis. HPLC procedure for BPA quantification was performed as previously described (35). Briefly, the reversed-phase HPLC method exploits BPA derivatization with OPA and a spectrofluorimetric detector. The lowest BPA detection limit of this approach is 0.5 pmol.

Statistical analysis

Experiment 1. Statistical evaluation of the effect of L-DOPA preloading was made by analysis of variance (ANOVA) to test for differences among groups (Conditions 1 to 5). Then, a *post hoc* analysis, using the Student-Newman-Keuls method [SKN test], was applied for between group comparisons at a significant level of $p < 0.05$ when a significant difference was detected by ANOVA.

Experiment 2. The means and SD were computed for BPA concentrations measured from the following samples obtained from each studied animal: (1) tumor tissue; (2) normal brain tissue, collected in both cerebral hemispheres (ipsi- and contralateral to C6 glioma cells implantation); (3) blood. A Student *t* test for nonpaired data was employed to investigate group differences between those animals which received L-DOPA pretreatment and those animals that did not. To correct for multiple comparisons and to minimize the risk of type II errors, only p values ≤ 0.001 were considered statistically significant).

RESULTS

Experiment 1

In Fig. 1, mean (\pm SD) percentage differences between σ_p values measured in test and in reference samples are reported for each condition. The ANOVA analysis including the five experimental conditions (described earlier) revealed the presence of significant differences among them. SKN test demonstrated significantly higher σ_p percentage difference values in Condition 5 (BPA addition after 4-hour L-DOPA incubation) compared with any other condition. Moreover, significantly higher σ_p percentage difference values were found in Condition 3 (4-hour L-DOPA incubation) compared with Condition 2 (2-hour L-DOPA incubation). To exclude that the σ_p percentage difference observed in Condition 5 was the result of an independent accumulation of L-DOPA and BPA at two different times, we performed the following additional comparison: [mean σ_p percentage difference observed in Condition 5] vs [(σ_p percentage difference observed in Condition 1) + (σ_p percentage difference observed in condition 3)]. The statistical result showed significantly higher σ_p percentage values in Condition 5 than in Condition 1 and Condition 3.

Experiment 2

All rats survived the surgical implantation of C6 glioma cells. As shown by MRI data collected 5 days after operation (baseline), all animals developed a brain tumor with an average diameter of 1 mm. Longitudinal MRI monitoring demonstrated tumor growth in all rats. On average, tumor lesions reached a diameter ≥ 2 mm 12 days after surgical

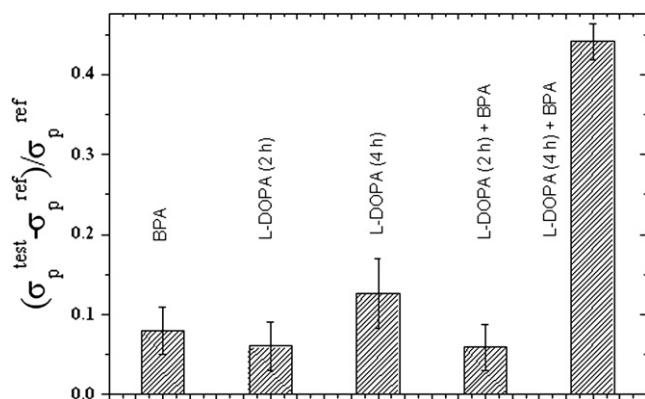


Fig. 1. Effects of L-DOPA preloading on borylphenylalanine (BPA) intracellular accumulation in C6 glioma cells. Percentage changes in cytosol electrical conductivity (σ_p) obtained from C6 glioma cells under five experimental conditions: (1) cells incubated with addition of 2 mmol BPA (first column on the left); (2) cells incubated with addition of 50 $\mu\text{g}/\text{mL}$ of L-DOPA for 2 hours (second column from the left); (3) cells incubated with addition of 50 $\mu\text{g}/\text{mL}$ of L-DOPA for 4 hours (third column from the left); (4) cells incubated with addition of 2 mmol BPA after a 2-hour preincubation with 50 $\mu\text{g}/\text{mL}$ L-DOPA (forth column from the left); (5) cells incubated with addition of 2 mmol BPA after a 4-hour preincubation with 50 $\mu\text{g}/\text{mL}$ L-DOPA BPA (fifth column from the left). Values are expressed as mean (\pm SD) percentage difference of σ_p between each test sample (C6 glioma cells under a specific experimental condition) and the reference samples. Significantly higher σ_p percentage difference values were observed in Condition 5 (BPA addition after 4-hour L-DOPA incubation) compared with any other experimental condition, and in Condition 3 (4-hour L-DOPA incubation) compared with Condition 2 (2-hour L-DOPA incubation). Group comparisons did not show any additional significant difference. See text for further details.

implantation (range, 10–14 days). The mean (SD) tumor volume assessed on T2-w scans from all the studied rats was $55.8 (8.3) \times 10^{-3}$ mL.

BPA concentrations assessed by HPLC from each sampled tissue (tumor, normal brain, blood) are summarized in Table 1 for the two experimental groups: rats that received BPA infusion only (Group A) and rats that received L-DOPA pretreatment before BPA infusion (Group B). BPA accumulation in tumor samples was significantly higher in Group B compared with Group A ($p < 0.0001$). Conversely, no significant difference was found in normal brain (sampled in both cerebral hemispheres) and blood samples between the two animal groups.

DISCUSSION

In this work, we tested the hypothesis that L-DOPA has an active and selective role in increasing the intracellular accumulation of BPA in C6 glioma cells. The results obtained *in vitro* demonstrate that L-DOPA promotes the cellular uptake of BPA, and extend findings previously observed in 9L gliosarcoma cells (31). As suggested by other authors (31), such an effect is likely to be related to mechanisms of active membrane transport, which are triggered by specific conditions. Indeed, our experiments showed that the σ_p

Table 1. BPA accumulation in sample of tumor tissue, normal brain, and blood from C6-glioma animal models with and without L-DOPA pretreatment

Sample	Mean \pm SD BPA/tissue concentration ($\mu\text{g}/\text{g}$)	
	Group A ($n = 10$)	Group B ($n = 15$)
Tumor	33.5 ± 7.5	88.3 ± 12.1
Normal brain (ipsilateral)	12.0 ± 5.2	10.6 ± 6.2
Normal brain (contralateral)	7.4 ± 2.2	4.8 ± 2.1
Blood	5.0 ± 1.8	4.8 ± 2.1

Abbreviations: BPA = borylphenylalanine; Group A = animals that received BPA infusion only; Group B = animals that received L-DOPA administration 24 hours before BPA infusion.

BPA concentration assessed by high-performance liquid chromatography from the sampled tissues (tumor, normal brain, blood) obtained from the C6 cells glioma rat model under two experimental conditions: BPA infusion (Group A); BPA infusion with L-DOPA pretreatment (Group B). Normal brain tissue was sampled from both hemispheres, ipsilateral and contralateral to glioma cells implantation. BPA concentration in tumor samples was significantly higher in condition B than in condition A. Conversely, no significant difference was found in normal brain and blood samples between the two groups.

changes resulting from the single addition of L-DOPA or BPA to cell cultures, is lower than that observed when the two compounds are given in combination (Fig. 1). Moreover, the latter effect is dependent on timing modalities by which the two compounds are administered. L-DOPA preloading increased 5 times the BPA intracellular accumulation with 4-hour incubation, whereas no significant change was observed with 2-hour preloading (Fig. 1). These findings indicate that the enhancing effect of L-DOPA preloading is based on events occurring in a relatively long time. Previous work has proposed that both BPA and L-DOPA penetrate through the cell membrane using two main mechanisms: by diffusion (slow process, essentially driven by concentration gradients) and by active carriers (fast process). Previous experiments, using mouse melanoma cells, suggested the presence of specific membrane antiport carriers with an high affinity for L-substrates, such as L-tyrosine, L-BPA, and L-DOPA (31). The activation of these carriers is supposed to be driven by a chemical gradient of L-molecules across cell membranes, due to their preliminary accumulation inside the cytosol (31). Using our results, we can speculate that 4 hours are necessary to reach in C6-glioma cells, by slow diffusion, a critical intracellular concentration of L-DOPA to trigger the faster L-antiport system. This explains why a massive accumulation of BPA was observed in C6 glioma cells only after 4-hour L-DOPA preloading.

The most striking findings of this work are the convergent results obtained with C6 glioma cells *in vitro* and using the equivalent animal model. L-DOPA preadministration produced in the rat model an enhancement of tumor BPA accumulation, which was about 2.7 times higher than in the control condition (Table 1). In clinical application, one of

the main limitations for BNCT effectiveness is the insufficient accumulation of ^{10}B carrier into the tumor cells. In this perspective, our results are particularly encouraging and might be considered for future BNCT clinical trials in humans. When comparing BPA concentration in blood and normal brain, there was no significant difference between rats that received L-DOPA and rats that did not. This makes the potential use of L-DOPA in BNCT of brain tumors even more attractive. Indeed, these results show the potential ability of L-DOPA to induce a significant increase of BNCT effectiveness (i.e., tumor cells disruption) without remarkable side effects (i.e., normal brain tissue damage) associated. The selective effect of L-DOPA on tumor tissues is likely to be due to genetic abnormalities in C6-glioma cells, conditioning an overexpression of antiport membrane carriers for L substrates.

Thus far, other strategies have been proposed to improve BNCT effectiveness by increasing BPA or BSH tumor accumulation. Some authors employed agents such as Cereport (RMP-7) (40) or mannitol (40, 41) to induce BBB disruption in different tumor models. Other researches focused on different modalities of BPA administration, demonstrating higher BPA concentrations in the L9-glioblastoma model after a prolonged intracarotid BPA infusion (6 hours vs 2

hours) (42, 43). These alternative strategies showed an increase of indexes such as tumor:brain and tumor:blood BPA concentration, suggesting a potential usefulness for BNCT. However, their potential toxicity has not been fully investigated. Our study showed a remarkable and selective increase of BPA uptake in tumor tissues using L-DOPA, which is a well-tolerated medication associated with modest side effects and low toxicity.

To the best of our knowledge, there are no published studies that have tested *in vivo* the effect of L-DOPA on BPA uptake in brain tumors. Our results appear promising, especially for their potential application to clinical therapeutic protocols. Nevertheless, this study has several limitations, such as the lack of determination of L-DOPA and BPA concentrations from blood, tumor, and normal brain samples at different time points and the absence of assessment of the radiation effects. Further animal studies are therefore needed not only to investigate further the effect of L-DOPA pretreatment on tumor BPA accumulation but also to evaluate the final result that may be obtained after BNCT radiation. In particular, appropriate protocols should be defined in terms of modalities of L-DOPA and BPA administration (i.e., doses and timing). These insights might eventually promote pilot studies in patients with malignant gliomas who are eligible for BNCT.

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