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Boronophenylalanine uptake in C6 glioma model is dramatically increased by L-DOPA preloading

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ABSTRACT

One of the main limitations for BNCT effectiveness is the insufficient intake of ^{10}B nuclei within tumour cells. This work was aimed at investigating the use of L-DOPA as enhancer for boronophenylalanine (BPA) uptake in the C6 glioma model. The investigation was first performed *in vitro*, and then extended *in vivo* to the animal model. BPA accumulation in C6 glioma cells was assessed, using radiowave dielectric spectroscopy (RDS), with and without L-DOPA preloading. C6 glioma cells were also implanted in the brain of 25 rats, randomly assigned to two experimental branches: (1) intra-carotid BPA infusion; (2) intra-carotid BPA infusion after pre-treatment with L-DOPA, administered 24 h before BPA infusion. All animals were sacrificed, and assessment of BPA concentrations in tumour tissue, normal brain, and blood samples was performed using high performance liquid chromatography (HPLC). L-DOPA preloading induced a massive increase of BPA concentration either *in vitro* on C6 glioma cells or *in vivo* in the animal model tumour. Moreover, no significant difference was found in the normal brain and blood samples between the two animal groups. This study suggests the potential use of L-DOPA as enhancer for BPA accumulation in malignant gliomas eligible for BNCT.

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1. Introduction

BNCT (Barth et al., 1999; Yamamoto et al., 2008) represents a promising adjuvant therapy for malignant gliomas. Previous Phase I and Phase II studies have consistently demonstrated no severe effects of BNCT related toxicity, and some preliminary evidence of therapeutic effectiveness (Busse et al., 2003; Diaz, 2003; Joensuu et al., 2003; Palmer et al., 2002). The major limitations for BNCT effectiveness are: the insufficient incorporation of ^{10}B into the tumour cells, even considering the most advanced methods of ^{10}B administration (Barth et al., 2002; Morris et al., 2002), and the relatively low specificity of ^{10}B uptake in tumour cells as compared to normal tissues. The most used ^{10}B carrier for clinical purposes is the *p*-dihydroxyboryl-phenylalanine

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$\text{C}_9\text{H}_{12}\text{BNO}_4$ (BPA). BPA is believed to pass through the BBB and cell membranes, and it is found at higher concentration in tumour cells. The details of the uptake mechanisms for BPA into tumour 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 tumour cells (Papasprou et al., 1994; Wittig et al., 2000). Moreover, BPA accumulation in 9L rat gliosarcoma cells is enhanced by either pretreatment with molecules targeted by L or A amino acid transport systems. These findings suggest that such transporters work by a substrate coupled antiport (exchange) mechanism, which is enhanced by preloading of specific amino acids. L-DOPA is a well known molecule whose chemical structure is similar to that of L-tyrosine and BPA. L-DOPA preloading has been previously demonstrated to improve several times the intracellular accumulation of BPA in 9L rat gliosarcoma cells (Wittig et al., 2000). 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 for their impact on potential clinical applications. The current work is focused on investigating the potential role of L-DOPA in BNCT (Capuani et al., 2008). 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 tumour cells implantation. Specific aims of this study were: (A) to replicate in C6 glioma cells the findings previously described in 9L gliosarcoma cells, by demonstrating a significant increase of BPA intracellular accumulation due to L-DOPA preloading (experiment 1); (B) to assess *in vivo*, using the C6 glioma cell rat model, the effect of L-DOPA preloading on BPA accumulation in tumours as compared to normal brain tissue (experiment 2). Experiment 1 was conducted using radiowave dielectric spectroscopy (RDS), which allows the electrical conductivity of the intracellular medium (cytosol) (σ_p) to be measured. Changes of σ_p have been shown as proportional to variations in intracellular BPA content (Capuani et al., 2002). 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 (Di Piero et al., 2000).

2. Methods and materials

2.1. *In vitro* experiment

Experiment 1: A reference sample of C6 glioma cells was first generated to assess the basic characteristics of permittivity and σ_s . Then, equivalent samples were produced to investigate changes of σ_p under five different experimental conditions: (a) cells incubated with addition of 2 mmol BPA; (b) with addition of 50 $\mu\text{g}/\text{ml}$ L-DOPA for 2 h; (c) with addition of 50 $\mu\text{g}/\text{ml}$ of L-DOPA for 4 h; (d) with addition of 2 mmol BPA after a 2 h pre-incubation with 50 $\mu\text{g}/\text{ml}$ L-DOPA; and (e) with addition of 2 mmol BPA after a 4 h pre-incubation with 50 $\mu\text{g}/\text{ml}$ L-DOPA. RDS was used to assess σ_p in each condition. This parameter is influenced by the intracellular concentration of BPA and/or L-DOPA. Conductivity cytosol changes were expressed 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}) (Fig. 1).

2.2. *In vitro* experiment

Experiment 2: Twenty five male Wistar rats weighing 300–350 g were used for the current experiment. Each rat underwent a stereotaxic brain implantation of about 10^6 deantigenized C6 glioma cells. Using magnetic resonance imaging (MRI) at 7 T, tumour implantation was first assessed 5 days after surgery (baseline), and tumour growth was followed up by serial longitudinal scans (one every 4 days). When the tumour size reached a minimum diameter of 2.0 mm, 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 ($N = 15$) received 50 mg/kg L-DOPA intra-peritoneally 24 h before BPA administration. Then each rat was anesthetized again, and injected in its right internal carotid 300 mg/Kg BPA–fructose complex. Animals belonging to the control branch ($N = 10$), underwent the same procedure with no preadministration of L-DOPA. All animals were sacrificed 150 min after BPA infusion. Tumour tissue, normal brain, and blood

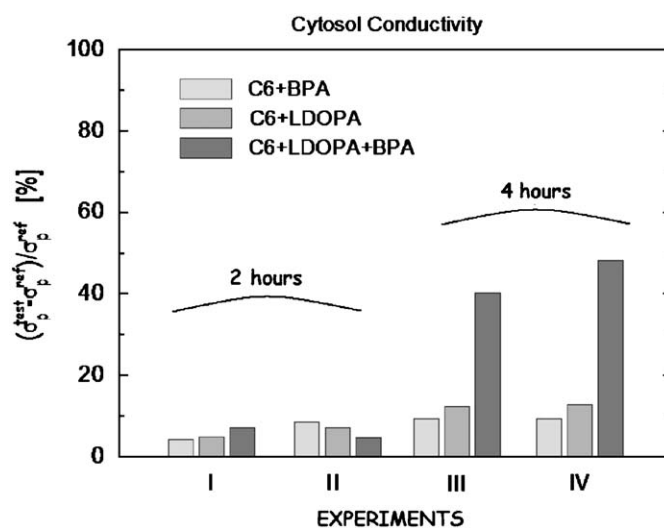


Fig. 1. Effects of L-DOPA preloading on BPA intracellular accumulation in C6 glioma cells obtained *in vitro*. Percentage differences between σ_p values measured in test (σ_p^{test}) and in reference samples (σ_p of C6 cells only σ_p^{ref}) are reported for C6 glioma cells under three experimental conditions: (a) cells incubated with addition of 2 mmol BPA (first column on the left); (b) cells incubated with addition of 50 $\mu\text{g}/\text{ml}$ of L-DOPA (second column from the left); and (c) cells incubated with addition of 50 $\mu\text{g}/\text{ml}$ of L-DOPA and then with 2 mmol BPA (third column from the left). In experiments I and II, L-DOPA was incubated for 2 h while in experiments III and IV it was incubated for 4 h.

samples were collected for HPLC quantification of BPA (Di Piero, et al., 2000).

3. Results

Experiment 1: In Fig. 1, percentage differences between σ_p values measured in test and in reference samples are reported for each condition and for repeated experiments (I–IV). Statistical analysis demonstrated significantly higher σ_p percentage difference values in the condition C6+L-DOPA+BPA after 4 h L-DOPA incubation as compared to any other condition. Moreover, significantly higher σ_p percentage difference values were found in the condition C6+L-DOPA+BPA after 4 h L-DOPA incubation compared to the condition C6+L-DOPA+BPA after 2 h L-DOPA incubation.

Experiment 2: Longitudinal MRI scans, reported in Fig. 2, showed that, on average, tumour lesions reached a diameter ≥ 2 mm 12 days after surgical implantation. BPA concentrations assessed by HPLC from each sampled tissue (tumour; normal brain; blood) are summarized in Fig. 3 for the two experimental groups: rats which received BPA infusion only; rats which received L-DOPA pre-treated before BPA infusion. BPA accumulation in tumour samples was significantly higher in the group pre-treated with L-DOPA compared to the control group ($p < 0.0001$). Conversely, no significant difference was found in normal brain and blood samples between the two animal groups.

4. Discussion

The results obtained *in vitro* demonstrate that L-DOPA promotes the cellular uptake of BPA, and extend findings previously observed in 9L gliosarcoma cells (Wittig et al., 2000). Such an effect is likely to be related to mechanisms of active membrane transport, which are triggered by specific conditions. Our experiments showed that the σ_p changes resulting from the single addition of L-DOPA or BPA to cell cultures are much lower

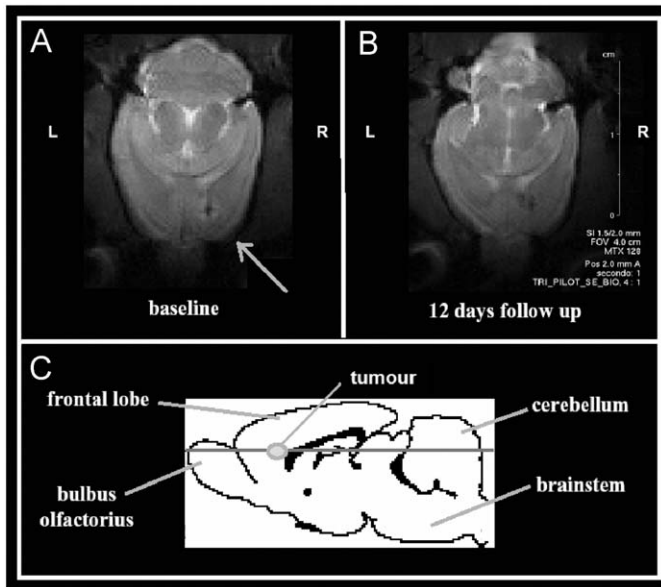


Fig. 2. Upper panels: axial T2-weighted MR images obtained from the same animal 4 days after tumour implantation (baseline, panel A) and at 12 days follow up (panel B). Baseline scan demonstrates the successful implantation of the C6 glioma cells that results in tumour development (see arrow). In panel C, a sagittal anatomical representation of the rat brain is shown. The grey circle represents the tumour location and the dark grey line corresponds to the slice shown in upper panels.

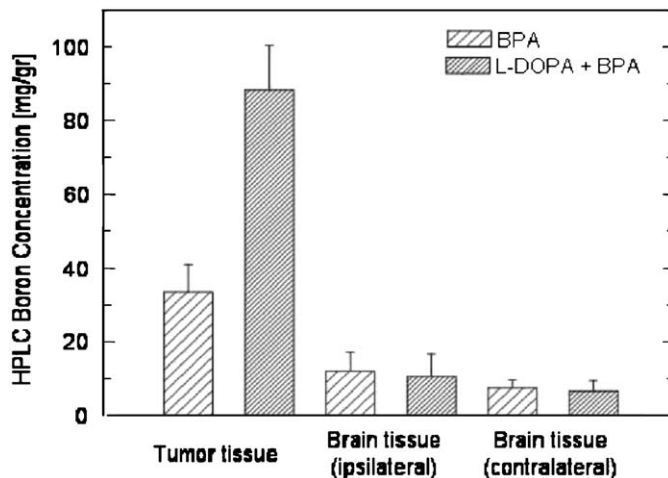


Fig. 3. BPA concentration assessed by HPLC from the sampled tissues (tumour; normal brain) obtained from the C6 cells glioma rat model under two experimental conditions: BPA infusion (BPA) and BPA infusion with L-DOPA pretreatment (L-DOPA+BPA).

than those observed when a 4 h L-DOPA preloading preceded BPA administration. It has been previously 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 a high affinity for L-substrates, such as L-tyrosine, L-BPA, and L-DOPA (Wittig et al., 2000). The activation of these carriers is supposed to be driven by a chemical gradient of L-molecules across cell membranes (Wittig et al., 2000). Our results suggest that 4 h are needed to reach in C6-glioma cells, by slow diffusion, a critical intracellular concentration of L-DOPA to trigger the faster

L-antiport system. The most striking findings of the present work are the convergent results obtained with C6 glioma cells *in vitro* and using the equivalent animal model *in vivo*. L-DOPA preadministration produced in the rat model an enhancement of tumour BPA accumulation, which was 2.7 times higher than in the control condition (Fig. 3). In clinical application, one of the main limitations for BNCT effectiveness is the insufficient accumulation of ^{10}B carrier into the tumour cells. In this perspective, our results are particularly encouraging for planning future BNCT clinical trials in humans. When comparing BPA concentration in blood and normal brain, there was no significant difference between rats which received L-DOPA and rats which did not. This makes the potential use of L-DOPA in BNCT of brain tumours even more attractive. Indeed, the potential ability of L-DOPA to induce a significant increase of BNCT effectiveness (i.e., tumour cells disruption) seems not to be associated with potential side effects (i.e., normal brain tissue damage).

5. Conclusion

In conclusion, the current study indicates a remarkable and selective increase of BPA uptake in tumour but not in normal brain tissue using L-DOPA, which is a well known medication. Reported data demonstrate the effectiveness of L-DOPA preloading to raise intratumoral BPA concentration to the requested values for a valid BNC therapy, disclosing in our opinion a translation of this method in the near future to clinical application.

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