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Evaluation of unnatural cyclic amino acids as boron delivery agents for treatment of melanomas and gliomas

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Abstract

Unnatural cyclic amino acids (UNAA) are a new class of boron delivery agents that are in a pre-clinical stage of evaluation. In the present study, the biodistribution of racemic forms of the *cis*- and *trans*- isomers of the boronated UNAA 1-amino-3-boronocyclopentanecarboxylic acid (ABCPC) and 1-amino-3-boronocycloheptanecarboxylic acid (ABCHC) was studied in B16 melanoma bearing mice and this was compared to *L-p*-boronophenylalanine (BPA). Boron concentrations were determined by inductively coupled plasma-optical emission spectroscopy (ICPOES) at 2.5 hrs following intraperitoneal (i.p.) injection of the test agents at a concentration equivalent to 24 mg B/kg. While all compounds attained comparable tumor boron concentrations, the tumor/blood (T/Bl) boron concentration ratios were far superior for both *cis*-ABCPC and *cis*-ABCHC compared to BPA (T/Bl = 16.4, and 15.1 vs. 5.4). Secondary ion mass spectrometry (SIMS) imaging revealed that the *cis*-ABCPC delivered boron to the nuclei, as well as the cytoplasm of B16 cells. Next, a biodistribution study of *cis*-ABCPC and BPA was carried out in F98 glioma bearing rats following i.p. administration. Both compounds attained comparable tumor boron concentrations but the tumor/brain (T/Br) boron ratio was superior for *cis*-ABCPC compared to BPA (6 vs. 3.3). Since UNAAs are water soluble and cannot be metabolized by tumor cells, they potentially could be more effective boron delivery agents than BPA. Our data suggest

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that further studies are warranted to evaluate these compounds prior to the initiation of clinical studies.

Keywords

Unnatural cyclic amino acids; melanomas; gliomas

1. Introduction

Boronated analogues of unnatural cyclic amino acids (UNAA's) belong to a new class of compounds that are gaining increasing attention as potentially more effective boron delivery agents for boron neutron capture therapy (BNCT) (Barth et al., 2012; Kabalka et al., 2001, 2011, 2004, 2003, 2006). The impetus for the design and synthesis of these compounds originated from the remarkable positron emission tomography (PET) observations in patients with the glioblastoma multiforme (GBM) and metastatic melanoma using fluorine-18 labeled boronophenylalanine (BPA) and carbon-11 labeled 1-aminocyclobutanecarboxylic acid. These studies revealed that the cyclic amino acids localized in these tumors at higher concentrations than BPA (Hubner et al., 1998; Nichols et al., 2002). The fact that they are water soluble and cross the blood-brain barrier (BBB) (Aoyagi et al., 1988) provided the stimulus to further evaluate them as delivery agents for BNCT. This was especially true because of their ability to target infiltrating tumor cells within normal brain (Chandra et al., 2013; Kabalka et al., 2001, 2004, 2006, 2009). In the present report we describe biodistribution studies that have been carried out with the racemic forms of the *cis* and *trans* isomers of ABCPC and ABCHC and compared them to BPA using the B16 murine melanoma and F98 rat glioma models (Barth et al., 2009).

2. Materials and Methods

2.1 Synthesis and purification of *cis* and *trans* 1-amino-3-boronocyclopentanecarboxylic acid (ABCPC) and of 1-amino-3-borono-cucloheptanecarboxylic acid (ABCHC)

The chemical structures of the *cis* and *trans* molecules are shown in Fig. 1. Only one enantiomer of each is shown but the molecules were isolated as racemic mixtures and the structures were confirmed by X-ray analysis. It should be noted that the labeling of these compounds was transposed in an earlier report relating to ABCPC (Kabalka et al., 2009). The synthesis of both compounds involved the preparation of hydantoin precursors (nitrogen heterocycles) which could be separated by crystallization. The *cis* isomer precipitated out whereas the *trans* remained in solution and it was subsequently isolated by evaporation of the solvent. The hydantoins then were hydrolyzed to yield the desired amino acids. However, unexpectedly the process generated ammonium chloride as a byproduct that complexed with the desired products. To remove NH₄Cl from them, the boronic acid functionality was converted to the corresponding insoluble trifluoro-borate, which did not form a complex due to its ionic character. The trifluoroborate derivatives were isolated by filtration and hydrolyzed back to the boronic acids by mixing them with alumina, microwaving the mixture, and then washing off the alumina. Final purification was achieved by column chromatography using an alumina column with water as the eluent. The chemical

structures of the *cis* and *trans* ABCHC are also shown in Fig. 1. Again, only one enantiomer of each is shown but the molecules were isolated as racemic mixtures. These were prepared from the corresponding hydantoin in a manner completely analogous to that used to synthesize the ABCPC compounds.

2.2 Biodistribution of ABCPC, ABCHC and BPA in B16 melanoma bearing mice

All of the animal studies described in this report were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and our protocol was approved by the Institutional Animal Care and Use Committee of The Ohio State University (Permit #: A-3261-01 and IACUC protocol number 2011A000003). B16 melanoma cells were implanted subcutaneously (s.c.) into the right dorsum of syngeneic female C57BL/6 mice. Approximately 10 -12 d later, when tumors had attained a diameter of ~1 cm, biodistribution studies were initiated. The *cis* and *trans* compounds of ABCPC and ABCHC were used as a mixture of their enantiomers, which were dissolved in phosphate buffered saline (PBS) for the biodistribution studies. BPA (Katchem Ltd, Prague) was converted to a fructose complex as previously described (Yang et al., 1996). Based on our previous studies with BPA (Barth et al., 1997; Yang et al., 1997) a dose equivalent to 24 mg boron/kg body weight (b.w.) of each compound was administered by i.p. injection to groups of four mice. They were euthanized 2.5 hr. post injection, which based on previous studies with BPA (Barth et al., 1997), yielded the maximum T/Br and T/BI boron concentration ratios. Blood and various organs and tissues were removed and processed for boron determinations by means of inductively coupled plasma-optical emission spectroscopy (ICP-OES).

2.3 SIMS imaging studies of *cis*-ABCPC in B16 melanoma bearing mice

Small portions of the B16 melanoma were frozen for correlative SIMS studies from mice that had received a mixture of L- and D- enantiomers of *cis*-ABCPC. As described in the previous section, this was carried out in order to determine the cellular and subcellular localization of the compounds. For SIMS analysis, 4 μm thick cryosections were attached to silicon wafers ($\sim 1\text{ cm}^2$), freeze-dried and sputter coated with a 10 \AA layer of Au/Pd for enhancing their electrical conductivity. A CAMECA IMS-3f ion microscope was used for SIMS analyses (Chandra et al., 2013, 2000). Pixel-by-pixel image quantification of boron signals ($^{11}\text{B}^+$) was achieved by the secondary ion $^{12}\text{C}^+$ carbon normalization approach and using the relative-sensitivity-factors (RSF) of boron isotopes to the $^{12}\text{C}^+$ tissue matrix signals (Ausserer et al., 1989; Chandra et al., 2000; Smith et al., 1996). The absolute boron concentrations determined by this approach were converted into estimated wet weight concentrations by assuming 85% cellular water content.

2.4 Biodistribution of *cis*-ABCPC and BPA in F98 glioma bearing rats

The F98 rat glioma (#CRL-2397, American Type Culture Collection, Manassas, VA) has been used in a wide variety of studies in experimental neuro-oncology (Barth et al., 2009). F98 glioma cells (10^4) were implanted stereotactically into the right caudate nucleus of syngeneic Fischer rats (Yang et al., 1996) and ~12 days later biodistribution studies were carried out. The racemic *cis*-ABCPC compound, as a mixture of L- and D- enantiomers, was

dissolved in PBS for administration and BPA was converted to a fructose complex. Both compounds were administered i.p. at a dose of 250 mg/kg b.w., corresponding to 12 mg/kg for BPA and 16 mg/B/kg for *cis*-ABCPC. The rats were euthanized 2.5 hrs later and the organs were removed and processed for boron determinations by ICP-OES.

3. Results

3.1 Boron biodistribution of *cis*-ABCPC, *trans*-ABCPC, and BPA in B16 melanoma bearing mice

Boron uptake values, as determined by ICP-OES, for tumor, brain and blood for racemates of the *cis* and *trans*-ABCPC compounds and BPA in B16 melanoma bearing mice are summarized in Table 1. Although the tumor boron values for *cis* and *trans*-ABCPC were equivalent, the T/B1 ratio of the former was 16.4 vs. 3.9 for the latter suggesting that the pharmacodynamics of the two compounds were different. Although the tumor uptake of BPA was higher than that of *cis*-ABCPC (28.6 vs. 16.4 µg/g), the blood value of *cis*-ABCPC was very low (1.0 vs. 5.3 µg/ml). Consequently, the T/B1 ratio of *cis*-ABCPC was 16.4 vs. 5.4 for BPA suggesting that the former is more rapidly cleared from the blood. These observations indicate that the racemic *cis*-ABCPC had desirable characteristics as a boron-delivery agent and these might be improved by further separating them into their L- and D-enantiomers.

3.2 Boron biodistribution of *cis*- and *trans*-ABCHC and BPA in B16 melanoma bearing mice

Boron uptake values for tumor, blood, liver, kidney, spleen and brain and for the racemic *cis* and *trans*-ABCHC compounds and BPA in B16 melanoma bearing mice are summarized in Table 2. This detailed biodistribution study revealed that the pharmacodynamics of the *cis* and *trans*-ABCHC are quite different. *Cis*-ABCHC was superior to the *trans*-compound in delivering boron to the tumor (31.7 vs. 13.9 µg/g). In a direct comparison to BPA, *cis*-ABCHC was comparable to BPA in the amount of boron delivered to the tumor, but it had a far greater T/B1 ratio (15.1 for *cis*-ABCHC vs. 5.4 for BPA, Table 2). Furthermore, the boron concentrations of *cis*-ABCHC in blood, kidneys and spleen were lower compared to BPA indicating faster clearance while maintaining higher tumor boron concentrations, which were comparable to BPA. The more rapid blood clearance of *cis*-ABCHC also was consistent with observations relating to *cis*-ABCPC, as discussed above, and this would be a desirable property of these compounds as clinical boron delivery agents (Barth et al., 2012).

3.3 Single cell boron imaging SIMS studies of *cis*-ABCPC in B16 melanoma bearing mice

In contrast to ICP-OES determinations of boron concentrations of the test compounds, SIMS imaging for boron provides data on their subcellular localization. As shown in the frozen section photomicrograph (Fig. 2A) of a hematoxylin and eosin (H & E) stained section, the B16 melanoma was composed of a monomorphic population of cells with hyperchromatic nuclei and cytoplasmic melanin. SIMS analyses were made in adjacent cryosections and revealed the boron distribution from *cis*-ABCPC in the B16 melanoma cells. In positive secondary ion SIMS images of ³⁹K and ¹¹B, individual tumor cells were discernible (Fig. 2B). Boron was localized in nuclei and cytoplasm of B16 cells with discernible

heterogeneity (Fig. 2C). The regions of interest (ROI) in each SIMS image represented approximately 10-15 individual cells, taken together for quantitation of boron by SIMS images. As determined by SIMS from 47 ROIs in 7 SIMS imaging fields, the boron concentration in tumor cells at 2.5 hrs was $14 \pm 5 \mu\text{g/g}$ (mean + SD). Both ICP-OES measurements (Table 1) and SIMS analyses revealed close agreement in boron concentrations in tumor and individual tumor cells at 2.5 hrs following administration. A plausible explanation for this close agreement can be attributed to the fact that most of the *cis*-ABCPC had cleared from the blood and extracellular fluids by 2.5 hrs and the intracellular *cis*-ABCPC was bound to matrix components.

3.4 Boron biodistribution studies of *cis*-ABCPC and BPA in F98 rat glioma model

F98 glioma cells (Yang et al., 1996) were implanted stereotactically into the right cerebral hemisphere of syngeneic Fischer rats and 12-14 d later they received either *cis*-ABCPC or BPA (24 B/kg b.w.) by i.p. injection. The rats were euthanized 2.5 hrs later and boron biodistribution was quantified by ICP-OES (Table 3). Both *cis*-ABCPC and BPA delivered comparable amounts of boron to the tumor, blood, kidneys and tumor bearing (ipsilateral) cerebral hemisphere. However, the contralateral, non-tumor bearing cerebral hemisphere had less boron (4.4 $\mu\text{g/g}$ boron for *cis*-ABCPC vs. 6.5 $\mu\text{g/g}$ for BPA, Table 3). This suggests that the uptake of *cis*-ABCPC in normal brain might be less compared to that of BPA, which would be a highly desirable property. A possible explanation as to why analysis of ipsilateral brain tissue, adjacent to the main tumor mass, did not reveal any significant differences between the compounds could have been due to the presence of infiltrative tumor cells in normal brain. Since ICP-OES boron measurements cannot differentiate between infiltrating tumor cells and normal brain tissue, they are a less accurate measure of the true boron content of normal brain.

4. Discussion and Conclusions

In the present study has demonstrated that racemic *cis*-ABCPC and *cis*-ABCHC compounds showed comparable boron uptake by B16 melanoma but a far superior T/BI boron concentration ratio was attained compared to that of BPA. Further separation of these compounds into their purified enantiomers might yield even better uptake data. Comparison of *cis*-ABCPC and BPA up-take data in the F98 rat glioma model provided evidence that the compound, even as a racemic mixture, was as effective as BPA in targeting the main tumor mass. The observation that the up-take of *cis*-ABCPC may be taken up in smaller amounts than BPA in normal brain deserves further investigation by SIMS imaging in order to better define the targeting of infiltrative tumor cells. This has been described in detail in a recently published report (Chandra et al., 2013). These types of unnatural amino acids have been shown to cross an intact BBB due to the presence of L-amino acid transporter (Aoyagi et al., 1988), which also is upregulated in high grade gliomas (Detta et al., 2009). Both of these characteristics may enhance the uptake of *cis*-ABCPC and *cis*-ABCHC by infiltrating tumor cells in the normal brain. Furthermore, both of these compounds are water soluble and cannot be metabolized, which may further enhance their retention in tumor cells compared to BPA, which is metabolized (Svantesson et al., 2002). Our observations suggest that further pre-clinical studies on enantiomerically pure *cis*-ABCPC and *cis*-ABCHC are

warranted to optimize their delivery and dosing and to evaluate their toxicity prior to clinical evaluation.

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Research Highlights

- Unnatural cyclic amino acids (UNAA) are a new class of boron delivery agents for neutron capture therapy
- ABCPC and ABCHC attained higher tumor/blood ratios *vs.* BPA in B16 melanoma bearing mice
- The tumor/brain ratio of *cis*-ABCPC was superior to BPA (6 *vs.* 3.3) suggesting that further studies are warranted

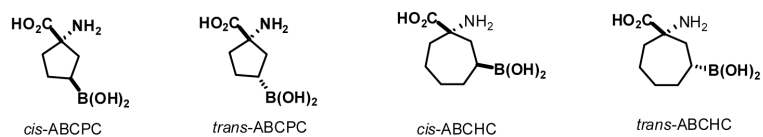


Fig.1. Structures of the UNAA used in the study. Although the molecules were used as racemates, only one enantiomer is drawn.

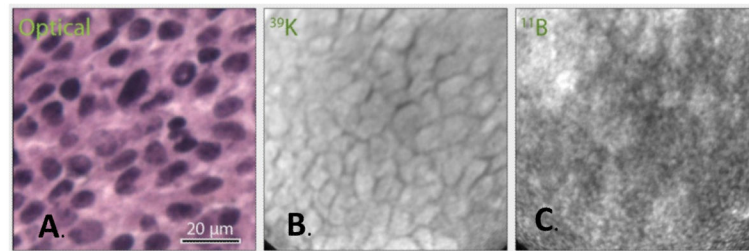


Fig.2. SIMS imaging analysis of B16 tumor from *cis*-ABCPC treated B16 melanoma bearing mice. The optical image was recorded from H&E stained 4 μm thick tumor cryosection. SIMS images were recorded from adjacent 4 μm thick cryo-sections. The SIMS $^{39}\text{K}^+$ image (B.) was integrated on the CCD camera for 0.2 sec and $^{11}\text{B}^+$ image (C.) for 2 min.

Table 1

Boron biodistribution of *cis*-ABCPC, *trans*-ABCPC and BPA in B16 melanoma bearing mice. Boron concentrations are expressed in ppm wet weight (mean \pm SD).

Compound ^a	Boron concentration ($\mu\text{g/g}$) ^b			Ratios	
	Tumor	Brain	Blood	T/Br	T/Bl
<i>cis</i> -ABCPC	16.4 \pm 9.0	4.1 \pm 3.5	1.0 \pm 0.9	4.0	16.4
<i>trans</i> -ABCPC	13.0 \pm 2.4	3.3 \pm 1.1	3.3 \pm 1.1	3.9	3.9
BPA	28.6 \pm 4.6	n.d.	5.3 \pm 0.8	n.d.	5.4

^a All compounds at a dose of 24 mg B equivalent/kg b.w. were administered i.p. and animals were euthanized at 2.5 hrs post-dosing.

^b Boron concentrations were determined by ICP-OES for groups of 4 mice. "n.d. is not determined.

Table 2Boron biodistribution of *cis*-ABCHC, *trans*-ABCHC, and BPA in B16 melanoma bearing mice.

Compound ^a	Mean Boron Concentration ($\mu\text{g/g}$ tissue) \pm SD ^b						Ratios	
	Tumor	Blood	Liver	Kidney	Spleen	Brain	T/Bl	T/Br
<i>cis</i> -ABCHC	31.7 \pm 11.2	2.1 \pm 0.9	3.6 \pm 2.1	5.8 \pm 1.6	4.1 \pm 1.5	2.6 \pm 0.6	15.1	12.2
<i>trans</i> -ABCHC	13.9 \pm 2.7*	3.2 \pm 0.6	4.7 \pm 1.0	8.3 \pm 3.2	5.3 \pm 1.4	n.d.	4.3	-
BPA	28.6 \pm 4.6	5.3 \pm 0.8*	5.3 \pm 1.0	18.9 \pm 1.3*	14.1 \pm 5.3*	n.d.	5.4	-

^a All compounds at a dose of 24 mg B equivalent/kg b.w. were administered i.p. and animals were euthanized at 2.5 hrs post-dosing.

^b Boron concentrations were determined by means of ICP-OES. Means and standard deviations were calculated for each group of 4 mice. "n.d." designates "not determined." Ratios of boron concentrations in tumor (T) to blood (Bl) and tumor to brain (Br) were calculated.

* Boron concentrations for the different compounds in each group of tissue and blood samples were compared using analysis of variance (ANOVA) and Tukey's *post hoc* test for multiple comparisons. A *p* value of less than 0.05 was considered significant. The tumor uptake value of *trans*-ABCHC was significantly different ($p < 0.05$) from *cis*-ACCHC and BPA.

Table 3Boron biodistribution of *cis*-ABCPC and BPA in F98 Glioma bearing Rats.

Test Agent ^b	Boron Concentrations ($\mu\text{g/g wt}$) \pm SD ^c					Ratios	
	Tumor	Ipsilat. Brain	Contralat. Brain ^d	Blood	Kidney	T/Bl	T/Br
<i>cis</i> -ABCPC	26.2 \pm 9.7	7.9 \pm 3.2	4.4 \pm 0.5 ^d	6.7 \pm 1.5	33.6 \pm 7.7	3.9	6.0
BPA	21.5 \pm 8.4	8.8 \pm 2.1	6.5 \pm 0.5	5.1 \pm 1.4	30.1 \pm 5.5	4.2	3.3

^aF98 glioma cells (10^4) were implanted into the right caudate nucleus of syngeneic Fischer rats.

^bThe test agents were administered i.p. at a dose of 250 mg/kg b.w. and rats were euthanized 2.5 hrs post-dosing.

^cBoron concentrations were determined by ICP-OES and mean and standard deviations were calculated from groups of 4 rats.

^dContralateral brain tissue contained significantly less ($p < 0.05$) boron in rats that received *cis*-ABCPC compared to BPA.