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Evaluation of TK1 targeting carboranyl thymidine analogues as potential delivery agents for neutron capture therapy of brain tumors

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Abstract

In this report we describe studies with N5-2OH, a carboranyl thymidine analogue (CTA) that is a substrate for thymidine kinase 1 (TK1), using the F98 rat glioma model. *In vivo* BNCT studies have demonstrated that intracerebral (i.c.) osmotic pump infusion of N5-2OH yielded survival data equivalent to those obtained with i.v. administration of boronophenylalanine (BPA). The combination of N5-2OH and BPA resulted in a modest increase in MST of F98 glioma bearing rats compared to a statistically significant increase with the RG2 glioma model, as has been previously reported by us (Barth et al., 2008). This had lead us to synthesize a second generation of CTAs that have improved *in vitro* enzyme kinetics and *in vivo* tumor uptake (Agarwal et al., 2015).

Keywords

carboranyl thymidine analogues; N5-2OH; F98 rat glioma; BNCT

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1. Introduction

Studies on the design and synthesis of boron-containing nucleosides as potential delivery agents for boron neutron capture therapy (BNCT) were initiated in the early 1990s by Soloway and his co-workers (Wyzlic et al., 1994; Soloway et al., 1998). More recently, Tjarks and his research team have designed and synthesized a second generation panel of 3-carboranyl thymidine analogues (3CTAs) as substrates of thymidine kinases (TK), and these are described in a review on the chemistry of the 3CTAs (Khalil et al., 2013). TK1 is widely distributed and expressed in all neoplastic cells, but it is virtually absent in normal cells. TK1 and TK2 catalyze the transfer of γ -phosphate groups from ATP to the 5'-hydroxyl groups of the respective nucleosides. The expression of TK1 is tightly regulated during the cell cycle, and the active enzyme is found only in S-phase cells (Arner and Eriksson, 1995; Persson et al., 1985). Enzyme kinetic and cell culture studies have been carried out on a selected number of 3CTAs designated N4, N5, and N7 and the corresponding 3-dihydroxypropyl derivatives, N4-2OH, N5-2OH and N7-2OH (Al-Madhoun et al., 2004; Barth et al., 2004). Of these compounds, N5-2OH (3-[5-{2-(2,3-dihydroxyprop-1-yl)-*o*-carboran-1-yl}pentan-1-yl]thymidine) (Fig.1) had the most favorable *in vitro* properties, which included high phosphorylation by TK1, low intrinsic toxicity to normal and malignant cells, and high, TK1-dependent cellular uptake and retention. Based on these properties, we carried out *in vivo* studies to evaluate N5-2OH as a boron delivery agent for BNCT using the RG2 rat glioma model (Barth et al., 2008) and in that study there was convincing evidence of therapeutic efficacy. A significant increase in mean survival time (MST) was seen in animals that had received intracerebral (i.c.) administration of N5-2OH compared to that obtained with animals that had received intravenous (i.v.) BPA (45.6 ± 7.2 d *versus* 35.0 ± 3.3 d). Furthermore, the combination of i.c. N5-2OH and i.v. BPA resulted in an increased MST of 52.9 ± 8.9 d. In the present report we describe our studies using another rat brain tumor model, the F98 rat glioma (Barth and Kaur, 2009), which has been used extensively by us to evaluate a variety of boron delivery agents for BNCT (Barth et al., 1997; 2000), and contrary to our previous report, there was only a modest increase in MST compared to irradiated controls.

2. Materials and Methods

2.1 Target validation

Target validation was established by means of Western blot analysis of TK1 protein expression in the F98 glioma and the L929 wildtype TK1⁺ and mutant TK1⁻ cell lines. Total cell lysates, prepared using T-PER tissue protein extraction reagent (ThermoScientific), were resolved by electrophoresis on sodium dodecylsulfate (SDS)-polyacrylamide gel (4–20%) and transferred on to polyvinylidene difluoride membranes (PVDF) (Bio-Rad). Western blotting was performed using TK1 primary antibodies (Novus Biologicals) and anti-rabbit-IgG horseradish peroxidase (HRP)-linked secondary antibodies (Cell Signaling). The signals shown in Fig. 2 were derived from proteins on the same membrane using SuperSignal West Dura Chemiluminescent Substrate (ThermoScientific). Subsequently, the membrane was incubated with anti-beta actin antibodies for protein loading. The signal

intensity of TK1 relative to actin for each cell sample was determined by ImageJ and EXCEL.

2.2 F98 glioma model, biodistribution, and BNCT studies

All animal studies were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, D.C., 1996) and the protocol was approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University. The F98 (CRL-2397 ATCC, Manassus, VA) rat glioma, which has been described in detail elsewhere (Barth and Kaur, 2009). N5-2OH and ^{10}B -enriched N5-2OH were synthesized as described previously (Al-Madhoun et al., 2002; Byun et al., 2006). Biodistribution studies and BNCT were carried out 14 d after intracerebral (i.c.) stereotactic implantation of either 10^4 glioma cells for the former or 10^3 cells for the latter. One week prior to irradiation the rats were shipped by air to the Massachusetts Institute of Technology (MIT) Nuclear Reactor Laboratory for irradiation at the MITR-II facility. Due to low tumor uptake of N5-2OH following i.v. administration, it was infused by means of Alzet[®] osmotic pumps (model #2001D, DURECT Corp., Cupertino, CA) over 24 hr at a flow rate of 8.33 $\mu\text{L/hr}$. Rats were randomized into experimental groups of 8–10 animals each as follows: (1) N5-2OH administered over 24 hr to the site of the brain tumor by Alzet[®] osmotic pumps, followed immediately thereafter by BNCT; (2) N5-2OH plus i.v. BPA and BNCT; (3) i.v. BPA and BNCT; (4) i.c. delivery of DMSO and BNCT; (5) untreated controls. Animals in Groups 1 and 2 received 500 μg of ^{10}B -enriched N5-2OH at the same concentration as that used in the biodistribution studies containing 100 μg of boron, solubilized in 35% DMSO in a volume of 200 μL . Rats were anesthetized with a mixture of ketamine and xylazine, following which they were irradiated for 5 min at the MITR-II reactor, as previously described by us (Yang et al., 2006) within 1 hour following termination of the infusion. Animals in Groups 2 and 3 received i.v. 500 mg of ^{10}B -enriched BPA (Katchem, Czech Republic), formulated as a fructose complex 2.5 hr prior to neutron irradiation. Parenthetically, it should be noted that direct i.c. administration of BPA did not increase the tumor boron uptake compared to that obtained following i.v. administration (Yang, W. and Barth, R.F., unpublished data).

2.3 Dosimetry and clinical monitoring

Dosimetric measurements were performed, as previously described (Yang et al., 2006). After completion of BNCT, the animals were held at MIT for ~ 3 d to allow induced radioactivity to decay before they were returned to Columbus, OH. All animals were weighed $3\times$ per week and their clinical status was evaluated at the same time. Once the animals had evidence of progressively growing tumors, as evidenced by a 20% loss in body weight over 3 d, they were euthanized in order to minimize discomfort (Barth et al., 2000). The brains of all animals in the therapy studies were removed after death, fixed in 10% buffered formalin and then cut coronally, and they were processed for neuropathologic examination to confirm that the animals had progressively growing tumors at the time of death. The mean survival time (MST), standard error (SE), and median survival times (MeST) were calculated for each group using the Kaplan-Meier method and Cox survival plots. Because proportional hazards were satisfied, pairwise Wald log rank tests were

performed comparing the Cox survival plots of the groups using a Bonferroni method of adjustment for multiple comparisons (Madsen and Moeschberger, 1986).

3. Results

3.1 Target validation, in vivo biodistribution studies and dosimetry in glioma bearing rats

As shown in Fig. 2, Western blots demonstrated that TK1 enzyme expression of F98 glioma and the wildtype L929 cell line was approximately 2X higher than the TK1(–) mutant cell line. In contrast, normal brain cells do not have amplified expression of TK1 during the S phase of the cell cycle. Boron concentrations in tumor, brain and blood of F98 glioma bearing rats (Table 1), following i.c. administration of N5-2OH, were carried out in separate groups of animals prior to the initiation of therapy studies. The mean tumor boron concentrations in rats that received i.c. N5-2OH within 1 hr following termination of the infusion was 17.3 ± 4.3 µg/g, and 2.5 hours later following i.v. administration of BPA the total boron concentration was 28.0 ± 4.5 . The mean normal brain boron value was 3.8 to 4.0 µg in the tumor bearing cerebral hemisphere. The blood boron levels were <0.5 µg for N5-2OH and 5.5 µg/g for BPA. The tumor to normal brain (T:Br) boron ratio in F98 glioma bearing rats that received N5-2OH was 34.6:1 and 2.8:1 in rats that received BPA. Based on these boron concentrations, the unweighted absorbed physical radiation doses were determined as previously described (Yang et al., 2006). The highest physical tumor radiation dose was 8.2 Gy in rats that had received the combination of N5-2OH and BPA. If dose modifying factors such as the relative biological effectiveness (RBE) or compound biological effectiveness (CBE) factors had been used, then the calculated radiation doses to the tumor could have been much higher.

3.2 Responses following BNCT

Survival data for F98 glioma bearing rats following BNCT are summarized in Table 2 and the corresponding Kaplan-Meier and Cox survival plots are shown in Fig 3A and 3B, respectively. The longest MST of 43.5 ± 5.9 d was observed in animals that received the combination of N5-2OH and BPA compared to 37.9 ± 6.8 d for rats that received N5-2OH alone and 36.7 d for animals that received BPA alone. As determined by means of the Cox proportional hazards model (Fig. 3B), the differences in the survival curves of animals that received i.v. BPA + N5-2OH *versus* N5-2OH alone was only of borderline significance ($p = 0.054$). However, there was no significant difference in the survival curves of rats that received i.v. BPA compared to N5-2OH ($p = 0.46$), or the combination compared to i.v. BPA alone ($p = 0.12$). The MSTs of irradiated control rats was 31.3 d, and 25.4 d for untreated controls. In the case of glioma bearing rats, the survival curves of animals that received either N5-2OH or BPA were not significantly different from each other ($p = 0.46$) and had equivalent MSTs (37.9 d *versus* 36.7 d). Microscopic examination of hematoxylin and eosin stained sections of representative samples of the brains of all F98 glioma bearing rats revealed invasive brain tumors with rare foci of necrosis and hemorrhage.

4. Discussion

We previously have reported that N5-2OH was effective as a boron delivery agent for BNCT in RG2 glioma bearing rats (Barth et al., 2008). The MSTs of rats that received i.c. N5-2OH alone or in combination with i.v. BPA were 45.6 ± 7.2 d and 52.9 ± 8.9 d, respectively, compared to 35.9 d for BPA alone and 28.1 d for irradiated controls. The purpose of the present study was to evaluate N5-2OH using another rat brain tumor model, the F98 glioma, which has been used extensively by us to evaluate varying dosing and delivery paradigms of sodium borocaptate (BSH) and BPA as boron delivery agents for NCT (Barth et al., 1997; 2000). As demonstrated by Western blot analysis (Fig 2) the F98 glioma strongly expressed the TK1 enzyme at a level approximately 2X higher than L929 TK1⁺ cells, thereby providing target validation. However, in contrast to our previously reported data using the RG2 glioma model, i.c. administration of N5-2OH, followed by BNCT, resulted in a MST that was identical to that obtained with i.v. administration of BPA and there was only a modest increase in the MST when i.c. N5-2OH was combined with i.v. BPA (43.5 d vs 37.9 d).

Recently reported *in vitro* studies (Sjuvarsson et al., 2013) have demonstrated that N5-2OH was effectively monophosphorylated in L929 TK1⁺ cells. However, non-phosphorylated N5-2OH was the major compound in both TK1⁺ and TK1⁻ cells. Since the F98 glioma strongly expressed TK1, there is no readily apparent explanation for the lack of effectiveness of N5-2OH in the F98 glioma model compared to its significant efficacy in both the RG2 glioma and TK1⁺ L929 tumor models (Barth et al., 2008). Kinase mediated trapping (KMT) appears to be the operative mechanism for the accumulation of N5-2OH in the TK1⁺ L929 and RG2 tumor cells (Barth et al., 2004; Barth et al., 2008; Sjuvarsson et al., 2013) and based on the similarity of the RG2 and F98 gliomas, one would have predicted the same for the latter.

The low water solubility of N5-2OH was one major limitation of this compound. To solve this problem Tjarks and his research team have synthesized second generation 3CTAs containing guanidino- and amidino groups in the tether between the carborane cluster and the thymidine scaffold (Agarwal et al., 2013 and 2015). These agents have improved water solubility but are inferior to N5-2OH as TK1 substrates. 3CTAs that had an amino group attached directly to the *meta*-carborane cage in the substituent at the 3-position of thymidine were superior substrates for TK1 than N5-2OH (Byun et al., 2005). The lack of therapeutic efficacy in the F98 glioma model raises an important question regarding the potential usefulness of N5-2OH as a boron delivery agent for NCT. Our data with the F98 glioma model provide a cautionary note to our previous report (Barth et al., 2008), which may have been overly optimistic. It was for this very reason that Tjarks and his research team embarked on the synthesis and evaluation of a second generation of CTAs. The most promising of these compounds, designated CT18A, was approximately 3 to 4X superior as a substrate and inhibitor of human TK1 than N5-2OH. Furthermore, following i.c. administration, it attained boron concentrations 3.7X greater than that of N5-2OH in RG2 glioma bearing rats (32.5 vs. 8.8 $\mu\text{g B/g tumor}$) (Agarwal et al., 2015).

Although not directly related to the studies described in the present report, *in vitro* studies were carried out with N5-2OH to evaluate it as a potential radioprotective or radiosensitizing agent using F98 glioma cells. This was determined by means of clonogenic assays using varying concentrations of N5-2OH (17.5–150 μ M) in combination with varying doses of 6 MV X-rays (2.5–15 Gy) using a linear accelerator (LINAC) as the radiation source. No significant changes were noted in the surviving fractions of F98 glioma cells pre-treated with N5-2OH for 24 hr prior to irradiation (Barth, R.F. and Nakkula, R.J., unpublished data).

In conclusion, *in vivo* BNCT studies with the F98 glioma model have demonstrated that i.c. delivery of N5-2OH yielded survival data that were equivalent to those obtained with i.v. administration of BPA and that the combination of N5-2OH and BPA resulted in only a modest increase in MST of F98 glioma bearing rats compared to the statistically significant increase previously reported in RG2 glioma bearing rats (Barth et al., 2008). Due to other issues which may not be so easily resolved, it is an open question where *in vivo* BNCT brain tumor studies with CTAs go from here. Since our ability to carry out *in vivo* BNCT studies with these compounds is no longer possible due to a variety of reasons, it may not be possible to evaluate their therapeutic efficacy in brain tumor models.

Acknowledgments

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Fig. 1.
Chemical structure of N5-2OH.

Fig. 2. Western blot analysis of TK1 protein expression in F98W, TK1⁻ L929, and TK1⁺ L929 cells.

Fig. 3.

A: Kaplan-Meier survival plots for F98 glioma bearing rats. Survival times have been plotted for untreated animals (●), irradiated controls (○), and animals that received i.v. BPA (◆) or N5-2OH either alone (▲) or in combination with BPA (◇). Fig. 3B: Cox survival plots for F98 glioma bearing rats. Survival times have been plotted for untreated animals (●), irradiated controls (○), and animals that received i.v. BPA (◆) or N5-2OH either alone (▲) or in combination with BPA (◇). It should be noted that Cox's method performs a simultaneous fit of the survival curves using all the data points with a partial likelihood approach. Therefore, the number of data points in each curve includes all of the death times, rather than those animals in a specific group.

Table 1

Biodistribution and physical radiation doses following intracerebral (i.e.) administration of N5-2OH to F98 glioma bearing rats

Group ^a	Boron uptake ($\mu\text{g/g}$) ^c				Physical dose (Gy) ^e	
	Tumor ^b	Brain ^d	Blood	Tumor/Brain Ratio	Tumor	Brain
F98/i.c. N5-2OH	17.3 $\pm$ 4.3	<0.5	<0.5	34.6	5.7	1.9
F98/i.c. N5-2OH + i.v. BPA	28.0 $\pm$ 4.5	4.0 $\pm$ 1.3	5.5 $\pm$ 1.5	7.0	8.2	2.7
F98/i.v. BPA	10.7 $\pm$ 1.7	3.8 $\pm$ 1.1	5.2 $\pm$ 1.3	2.8	4.2	2.6
F98/Irradiation control (i.c. of DMSO)	None	None	None	--	1.8	1.8

^a¹⁰B enriched N5-2OH was administered over 24 hr i.c. by means of ALZET[®] pumps at a flow rate of 8.33 $\mu\text{L/hr}$. BPA was administered intravenously 2.5 hr prior to BNCT.

^bF98 glioma cells were implanted into rats intracerebrally. The rats were irradiated 14 days after implantation.

^cBoron content was quantified by means of direct current plasma-atomic emission spectroscopy (DCP-AES). Boron uptake was present percent injected dose per gram tissue (ID%/g)

^dBoron concentrations for the tumor bearing cerebral hemisphere after excision of the tumor.

^ePhysical dose estimates include contributions from γ photons, ^{14}N (n,p) ^{14}C and ^{10}B (n, α) ^7Li reactions.

Table 2

Survival times of F98 glioma-bearing rats following CED of N5-2OH with or without i.v. BPA

Group ^a	No.	Survival time (days) ^b			% Increased life span ^c	
		Mean±SE	Median	Range	Mean	Median
i.c. N5-2OH + i.v. BPA	8	43.5±5.9	43.5	36-52	71	67
i.c. N5-2OH	9	37.9±6.8	38	29-51	49	46
i.v. BPA	10	36.7±3.2	37	32-42	44	42
Irradiated Controls	8	31.3±3.9	32	27-37	23	23
Untreated Controls	7	25.4±2.4	26	21-28	-	-

^a Mean and median survival times were determined for each group of 7-10 rats.^b Percent increased life span (%ILS) was defined relative to mean and median survival times of untreated controls.^c N5-2OH and BPA were administered as described in footnote in Table 1.