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A new Class of Drugs for BNCT? Borylated derivatives of ferrocenium compounds in animal experiments

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Abstract

A new class of drugs, borylated derivatives of ferrocenium compounds, which show a comparatively facile synthesis is investigated on their boron neutron capture accumulation. Investigations focused on the fast and effective testing of 12 ferrocene derivatives with tetracoordinated boron atoms, which should accumulate in rodent tumors. The macroscopic studies on time-dependent boron distributions and boron concentrations in mice were carried out by inductively coupled plasma-atomic emission spectrometry, inductively coupled plasma-mass spectrometry, and quantitative neutron capture radiography. The determination of boron concentrations after injection of **2b** showed high boron contents in spleen, liver, kidneys, less in lung and muscle, and poor in integral blood, blood plasma, tumor, and brain. It is interesting to note that **2b** penetrates the blood-brain barrier which may be advantageous in the treatment of astrocytomas and glioblastomas.

Introduction

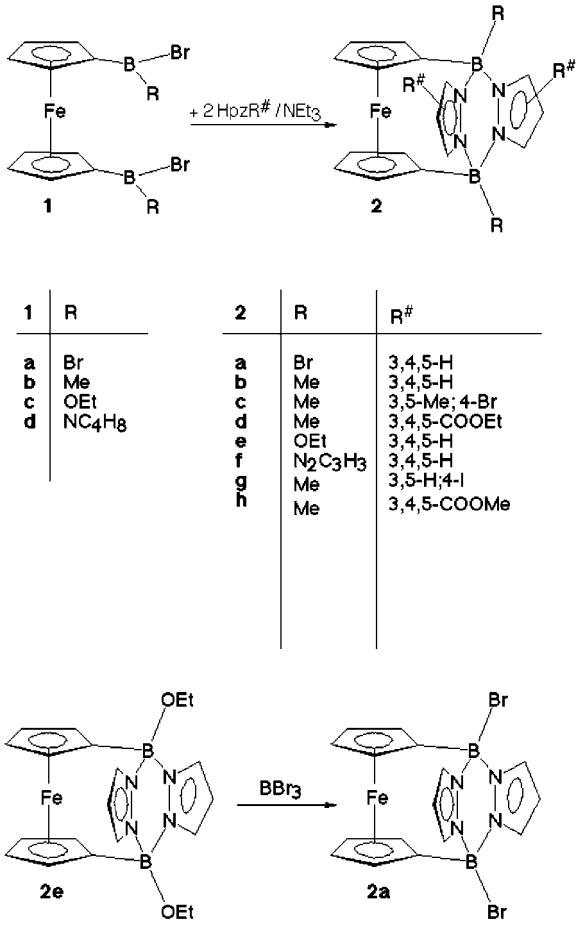
Surgery, radiotherapy and chemotherapy are the most common treatments against cancer, but the selective destruction of primary tumors by irradiation might fail due to radiosensitive tissues next to the neoplasm. To reduce the radiation dose to normal tissue while safe application of high radiation doses to malignant tumors in curative radiation therapy, treatment schemes are of special interest in which the tumor cells are selectively sensitized and subsequently destroyed by radiation. In principle, boron neutron capture therapy (BNCT) fulfils these requirements. BNCT is a bimodal form of radiation therapy against cancer (Locher 1936). In tumor tissue selectively accumulated boron-10 (^{10}B) followed by irradiation with thermal neutrons generates high energy $^{4}He^{2+}$- and $^{7}Li^{3+}$-particles. Due to their high linear-energy-transfer (LET) and the short range emission of these particles the radiation damage is confined to the vicinity of the boron-accumulating tissue (Coderre & Morris 1999; Gahbauer *et al*. 1998; Soloway *et al*. 1997). For tumor treatment, a concentration of approximately 10–30 μg ^{10}B per gram of tumor, which is equivalent

to 10^9 atoms of ^{10}B per cell, is required to destroy the tumor. BNCT is bound for treatment of carcinoma near the surface, i.e., approximately 2–5 cm, because the efficacy attained is due to neutron fluxes and neutron energies generated in nuclear reactors.

Clinical studies for treatment of glioblastoma and astrocytoma on BNCT are performed in the EU since 1997, Finland since 1999, Japan since 1968, and the United States since 1961 using L-dihydroxyborylphenylalanine hydrochloride as its water soluble fructose complex (BPA-F) and mercaptoundecahydrododecaborate (BSH), which are regarded among other substances as first generation compounds (Gahbauer *et al.* 1998; Hawthorne 1993; Soloway *et al.* 1997, 1998). It has been shown that these 2 compounds deliver stable concentrations of ^{10}B of approximately 30 μg g body weight over long observation periods but with limited selectivity towards tumor cells.

Hitherto, several working groups targeting at tumor specific structures have successfully synthesized numerous boron compounds (Gahbauer *et al.* 1998; Hawthorne 1993; Soloway *et al.* 1997, 1998). In preclinical studies, advanced agents, e.g., boron clusters connected to porphyrines, monoclonal antibodies, epidermal growth factors, nucleosides, and amino acids, are investigated either by cell culture experiments or by application of drugs into rodents to evaluate the effectiveness of new structures for BNCT. Especially, macroscopic investigations of boron distributions in small mammalians reveal toxicology, biodistribution, and accumulation of the administered substances in reasonable times and can be transferred to clinical studies (Probst 1999). The success or pitfalls of new synthesized compounds for BNCT can be effectively deduced from preclinical *in vivo* studies of biodistributions of the applied substances in rodents (Probst 1999).

With regard to the biological problems, we focused our investigations on fast and effective testing of 12 new boron-containing compounds which should accumulate in rodent tumors, using a class of ferrocenium derivatives as boron carriers in BNCT. 12 borylated ferrocene derivatives (Herdtweck *et al.* 1996; Jäkle *et al.* 1995, 1996), compare Figure 1 and Figure 2, were tested in C3H-mice bearing an AT17-mammary carcinoma. In previous work by Köpf-Maier *et al.* (1984, Angew. Chem. Int. Ed. Engl., J. Cancer Res. Clin. Oncol.) and Köpf-Maier & Köpf (1987), ferrocenium compounds have been acknowledged to accumulate in rodent tumors, which have been applied in doses of up to 500 mg kg body weight. Beside the aspect that these compounds likely qualify for BNCT, this class of drugs has also a cell killing, cytostatic effect (Köpf-Maier *et al.* 1984, Angew. Chem. Int. Ed. Engl., J. Cancer Res. Clin. Oncol.; Köpf-Maier & Köpf 1987). Moreover, boron is tetrahedrally coordinated in these ferrocene compounds which show a comparative facile synthesis and thus might be easily adapted to the various BNCT requirements. The macroscopic studies on time-dependent boron distributions and boron concentrations in mice after application of borylated ferrocenes were carried out by inductively coupled plasma-atomic emission spectrometry (ICP-AES), inductively coupled plasma-mass spectrometry (ICP-MS), and quantitative neutron capture radiography (QNCR) (Probst 1999; Probst *et al.* 1997).



1	R
a	Br
b	Me
c	OEt
d	NC_4H_8

2	R	$R^{\#}$
a	Br	3,4,5-H
b	Me	3,4,5-H
c	Me	3,5-Me; 4-Br
d	Me	3,4,5-COOEt
e	OEt	3,4,5-H
f	$N_2C_3H_3$	3,4,5-H
g	Me	3,5-H;4-I
h	Me	3,4,5-COOMe

Fig. 1. Scheme for synthesis of ansa-ferrocenes.

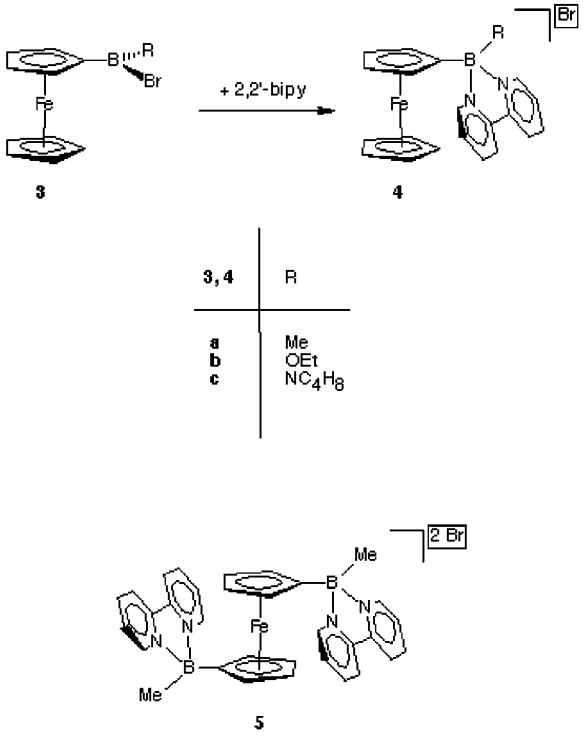


Fig. 2. Scheme for synthesis of bipyridine ferrocenium compounds.

Materials and methods

Drug syntheses and characterization

The key step of the synthetic pathway to compounds **2** is outlined in Figure 1. From 1,1′-bis-(dibromoboryl)ferrocene (1,1′-Fc(BBr_2)$_2$, **1a**), the starting materials **1b**, **1c**, and **1d** are accessible in one step by the reaction of **1a** with 2 equiv. of $Sn(CH_3)_4$, diethyl ether, or pyrrolidine/triethylamine, respectively. The synthesis of ferrocenophane **2a** was achieved in two different ways: (1) by adding 2 equiv. of pyrazole and 2 equiv. of NEt_3 in toluene to a toluene solution of **1a** at −78 °C [−108 °F] or (2) by a reaction of ethoxy-substituted ferrocenophane **2e** with 1 equiv. of BBr_3. Method 2 is preferable to method 1, because the product is obtained in high purity and very good yield of 87%. For the preparation of all other compounds **2**, it is advantageous to combine the appropriate ferrocenylborane and the required pyrazole derivative first and to add neat triethylamine afterwards. ^{11}B NMR, ^{1}H NMR, ^{13}C NMR, CI-MS, and X-ray structure analysis characterized the compounds, which are summarized in detail in Herdtweck *et al.* (1996) and Jäkle *et al.* (1995, 1996). All ferrocene derivatives **2** were transformed into the respective ferrocenium species by oxidation with $FeCl_3$ prior to application. Reaction of monoborylated ferrocenes **3a, 3b, and 3c** with 1 equivalent of 2,2′-bipyridine (bipy) gives the purple colored salts **4a, 4b, 4c**. The disubstituted compound **5** was obtained from **1b** and 2 equivalents of 2,2′-bipyridine, Figure 2, (de Biani *et al.* 1997). Dihydroxyborylferrocene **6** was isolated as a byproduct. All compounds administered *in vivo* are air stable and soluble in physiological NaCl solution.

Administration

The ferrocene derivatives were tested in C3H-mice bearing an isotransplanted AT17 mammary carcinoma. The AT17 mammary carcinoma is a well-characterized slow- growing adenocarcinoma induced by irradiation. It has a tendency to keratinize like human epithelial tumors. Even in tumors with large volumes necroses occur very rarely (Kummermehr 1985). The AT17 tumors were implanted subcutaneously into the right lateral abdominal wall (Weissfloch *et al.* 1997). For this purpose a sagittal incision of 5 mm in length has been performed on the skin and the tumor sample was placed caudal of the incision using a trocar. After having finished this procedure the cut was sealed by an adhesive (Histoacryl®; Braun, Melsungen, Germany). The mice were kept in an air-conditioned animal facility at 26 ± 2 °C [79 ± 4 °F], in cages containing five animals, each. The 12 hours day-night-cycle was controlled by a timer. Prior to the surgery, the mice were generally anaesthetized with 16 mg kg body weight xylazine (Rompun®, Bayer) and 100 mg kg body weight ketamine (Ketavet®, Parke Davis), dissolved in 0.9% NaCl. A volume of 0.20–0.25 ml of this cocktail was injected intraperitoneally (i.p.) (Flecknell 1998).

The ferrocenium derivatives were dissolved homogeneously in 0.9% NaCl using an ultrasonic bath. The mice received i.p. up to 400 mg kg body weight (11 mg boron kg body weight) of the derivatives diluted in 0.5 ml physiological saline, see Table 1. A solution of roughly 0.5 ml of volume was injected i.p.. After in parallel injection of the compounds in groups of four mice each at certain points of time, i.e., 0.5, 1.0, 1.5, 2.0, 4.0 h, the total of 20 mice were sacrificed. A total of 20 mice per compound were narcotized with ether, initially, and sacrificed by exsanguation for sampling the integral blood. A part of this blood was used to elaborate blood plasma by centrifugation. Thereafter, tumor, liver, kidneys, spleen, lungs, brain, were sampled *in toto*. Samples of the thigh muscles and the skin

Table 1. Administration data and veterinarian diagnosis; all drug concentrations are given in mg kg body weight

Substance	Concentration	Diagnosis
2b	400	Short-time ataxia, somnolence up to 12 h
	300	Reduced activity up to 40 min
	200-50[a]	Reduced activity up to 40 min less pronounced
2c[*]	400	Reduced activity, dyspnoea, somnolent
	200	Reduced activity up to 4 h
	100	Inconspicuous behavior ('fit'), no side effects
2e	400-100[b]	Reduced activity up to 8 h
2f	400-25[c]	Immediately heaviest excitations, jumps, polypnoea (panting breathing), ataxia soon followed by paraplegia of hint limbs, finally vomiting-like symptoms with gas bubbled bronchial secretion; euthanasia
2h[#]	400-25[d]	In the beginning short-timed screaming, rapidly becoming very somnolent, *e.g.* no reactions; probably cns depressant; euthanasia
4a	200	Immediate death post i.p.
	100	Death within less than 1 min, showing heaviest excitations
	50	Within couple of minutes dead, see 100
	25	Hyperactivity, followed by dyspnoea (laboured breathing), later on increasing somnolence; euthanasia
4b	400-25[c]	See 2f
4c	400-25[c]	See 2f
5	200-25[e]	Immediate death post/during i.p.
6	400-25[d]	Immediate excitations, followed by dys- and hypopnoea inclusively increasing somnolence; euthanasia

[*]: results obtained by injection of a suspension; [#]: foam formation during solubilization; [a]: stepwise 200; 100; 50; [b]: stepwise: 400/200/100; [c]: stepwise: 400/200/100/50/25; [d]: stepwise: 400/300/200/100/50/25; [e]: stepwise 200; 50; 20.

were removed. For all trials, blank samples were collected from untreated animals as control groups. The atropine protection was performed by application of 1 mg kg body weight atropine (Braun, Melsungen, Germany) diluted in 0.9% NaCl 10 min prior to the testing of compounds **2b, 2c, 2e, 4a** and **5**.

Boron analysis

After careful purification of solvents and standards and after microwave digestion of tissue samples, two independent analytical techniques, i.e., ICP-AES and ICP-MS, which were described in more detail in Probst *et al*. (1997) and Probst (1999), were employed for quantitative boron analysis in tissues. In addition, QNCR was used for investigation of whole-body cryosections of tumor bearing mice (Alfassi & Probst 1999).

ICP-AES

A sequentially operating ICP-AES plasma emission spectrometer Plasma 40/400 of Perkin Elmer equipped with a Ryton spray chamber, a quartz injector tube in the Fassel torch, and an autosampler AS-90 (Perkin Elmer) was used for the determination of boron concentrations. The sample aerosols are produced using a cross-flow nebuliser. Rh in a concentration of 10 μg ml was used as an internal standard. The operating conditions are summarized in more detail in Probst *et al*. (1997).

ICP-MS

The quadrupole ICP-MS ELAN 5000 of Perkin Elmer & SCIEX, which is equipped with a Pt-sampler, a Pt-skimmer cone, a Scott-type quartz glass chamber, a quartz injector tube in the Fassel torch, an autosampler AS-90 (Perkin Elmer), and an active film multiplier type AF 570 (ETP Pty Ltd, Ermington, Australia) is used for the presented experiments. The sample aerosols are produced using a Meinhard type C nebulizer or a micro concentric nebulizer MCN-100 (CETAC, Cheshire, U.K.), which was employed for small sample volumes. Boron was determined on the lines of 10 and 11 amu. A solution of a beryllium salt in a concentration of 10 ng ml was used as an internal standard for the ICP-MS measurements. The operating conditions are summarized in more detail in Probst *et al*. (1997).

Quantitative neutron capture radiography

The sacrificed mice were frozen at −72 °C [−98 °F] shaved, and embedded in 1.57% carboxymethyl cellulose. At −23 °C [−9 °F], whole-body sections of 50 μm thickness were cut by a cryotome and mounted on a boron-free tape together with the boron standards. The sections were freeze-dried at −72 °C [−98 °F] for 24 h, fixed on three different sheets of α-particle sensitive films (CR 39, Page Mouldings (Pershore) Ltd, England), introduced into PE bags, and evacuated. At the research reactor Studsvik, Sweden, the samples were exposed to a thermal neutron flux of 5.5×10^{12} n s cm^{-2} s^{-1}. For external calibration, human blood standards of enriched ^{10}B (95%) were prepared on each α-track etch. After etching in 6.25 n NaOH at 65 °C [149 °F] for 1.5 h, the films were evaluated first of all visually. The neutron capture radiography is described in more detail in Alfassi & Probst (1999) and Probst *et al*. (1997).

Results

In contrast to the numerous reagents with coordinated boron clusters (Hawthorne 1993; Soloway *et al*. 1997, 1998), a new class of drugs with boron as an integral component (Herdtweck *et al*. 1996; Jäkle *et al*. 1995, 1996) is investigated in this work. The drugs, which have been investigated on their BNC activity, consist in principle of three basic parts: (i) the metallocene, (ii) 1–2 tetrahedrally coordinated boron atoms, and (iii) 1–2 biologically active ligand systems. In this first tumor testing approach a ligand system of pyrazole derivatives **2a-h** were utilized as positional isomers of imidazole which is an integral component of the amino acid histidine as well as the DNA purine bases adenine and guanine, respectively. It is known that bipyridines selectively destroy the regular helically DNA stucture (Reichl 1997). Hence, borylated 2,2′-bipyridine ferrocenium compounds **4a-c** are tested on their ability to intercalate in DNA. It should be noted that arylborono acid derivatives, i.e., BPA and p-carboxyphenylborono acid, have been applied in BNCT successfully. Thus, dihydroxyborylferrocenium **6**, which combines ferrocenium with the borono acid function, were *in vivo* administered.

It should be emphasized that the whole testing procedure for one substance, i.e., tumor isotransplantation, time dependent drug administration, and quantitative boron analyses by ICP-AES and ICP-MS have been optimized to be carried out within 21 days. Parallel investigations of substances reduced the required effective time per substance even further.

Chemistry of the administered compounds

A variety of borylated ferrocene compounds have been obtained by the reaction of 1,1′-diborylferrocenes with selected pyrazole (Herdtweck *et al*. 1996; Jäkle *et al*. 1995, 1996), Figure 1, and bipyridine derivatives, Figure 2, from which 12 were investigated on *in vivo* distribution in tumor bearing mice, see Table 1. The introduction of a bridge spanning both cyclopentadienyl (Cp) rings prevents internal Cp rotation and increases the rigidity of the molecule. Thus, 1,1′-disubstituted ferrocenes can on principle be fixed in chiral conformations by interannular bridges. The X-ray structure of **2b** proves the bridged nature of the molecule with the central B_2N_4 ring possessing a boat conformation. Custom-tailored pyrazole derivatives, including chiral ones, are an easily accessible class of compounds. This versatility makes them particularly valuable building blocks and offers a facile way of synthesizing a large variety of ansa-metallocenes using always the same key step for the formation of the interannular bridge. In case a particular substituent at the pyrazole ring is not compatible with the highly reactive boron precursors **1a-d**, molecule **2c** offers the possibility to introduce this substituent after the formation of the *ansa*-metallocene moiety, when the boron centers are already tamed.

Studying the structure and dynamic behavior of *ansa*-ferrocenes with pyrazabole bridges by NMR investigations it was found that B-N bond cleavage occurs heterolytically and reversibly, and the energy of a B-N donor acceptor bond can be tuned over a wide range by choice of appropriate substituents at boron and nitrogen.

Toxicology, biodistribution, accumulation, and metabolism of the administered drugs

It should be noted that the B(R) bipy substituents of **4** and **5**, which might be released show *in vivo* toxicity due to unknown metabolites as well as from the similarity of the B(R) bipy fragment with the well-known herbicide Diguat®. Hence, it can be concluded that *in vivo* metabolism of the borylated ferrocene compounds might cause two problems: (i) separation of ferrocene or ferrocene derivatives from its boron-containing substituent and (ii) *ansa*-bridge opening in the case of **2**.

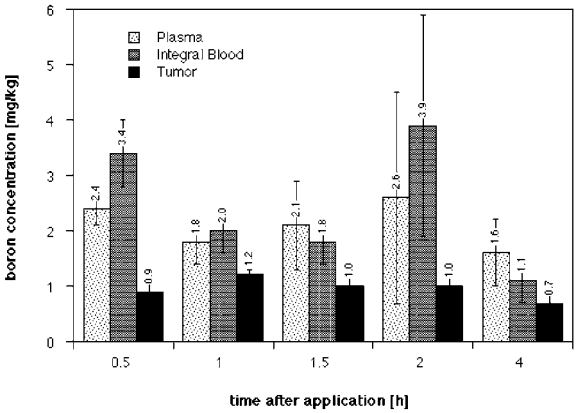


Fig. 3. Time-dependence of the boron concentrations in integral blood, blood plasma and tumor by administration of compund **2c**.

Shortly after the i.p. injections of up to 300 mg kg body weight (11 mg boron kg body weight) of the ferrocenium derivatives very different side effects were observed. All treated animals went through a short-term period of hyperactivity, see Table 1. The seven ferrocenium **2f, 2h, 4a, 4b, 4c, 5**, and **6** derivatives showed a very high neurological toxicity down to a drug concentration of 25 mg kg body weight, which could be seen in a dose-dependent rapid and significant change of the animal's behavior. For compounds **2b, 2c, 2e, 4a** and **5**, atropine protection was tested to decrease the observed side effects. These side effects did not occur during atropine protection, suggesting that these compounds inhibit the acetylcholin-esterase.

After injection of 300 mg kg body weight (11 mg boron kg body weight) **2b**, the mice became also somnolent, but recovered totally and returned to normal behavior after 40 min. A precision of 92% was obtained for ICP-AES and ICP-MS analysis of boron in a total of 79 samples. The determination of boron concentrations by ICP-AES and ICP-MS after injection of **2b** in parallel for 4 mice at each point of time showed high boron contents in spleen, liver, kidneys, less in lung and muscle, and poor in integral blood, blood plasma, tumor, and brain, see Table 2. No significant alterations of the mean boron concentrations independent from the time of application are observed when recognizing standard deviations. In more detail, the mean boron concentrations are in tumor (0.9 ± 0.1) mg kg, integral blood (2.4 ± 1.0) mg kg, and blood plasma (2.1 ± 0.4) mg kg (Figure 3). Hence, a mean tumor blood ratio of 0.36 results within a range of 0.26–0.64. Thus, it might be concluded that mean boron distributions in integral blood and blood plasma are approximately equal. The mean boron contents are in spleen (28 ± 7) mg kg, liver (20 ± 2) mg kg, kidneys (9 ± 1) mg kg, lungs (10 ± 3) mg kg, and muscle (6.0 ± 0.9) mg kg. Mean boron concentrations of (12 ± 4) mg kg in skin might result from muscle and blood residues. But it is remarkable that a mean boron content of (1.9 ± 0.4) mg kg was found in the brain. The boron concentrations of the alpha track etches were not quantified because it was obvious from a visual check as well as from ICP-AES and ICP-MS data that compound **2b** is not appropriate for BNCT.

Discussion

The AT17-tumor did not accumulate **2b** in the tumor tissue in amounts sufficient for BNCT. The observed toxicity of substances **2f, 2h, 4a, 4b, 4c, 5** and **6** might result from destruction and release of ferrocene or ferrocene derivatives which show both a cytostatic effect (Köpf-Maier *et al*. 1984, Angew. Chem. Int. Ed. Engl., J. Cancer Res. Clin. Oncol.; Köpf-Maier & Köpf 1987) and a high neurological toxicity. It should be emphasized that neurological toxicity of compounds **2b, 2c, 2e, 4a, and 5**, was inhibited completely by administration of atropine. Compound **2b** is interesting because of its penetration of the blood-brain barrier which is a characteristic that may be advantageous in the treatment of astrocytomas and glioblastomas. The modification of **2b** to reduce its toxicity, as well as further modifications of its administration will be a topic for research in the near future. To reduce the toxicity to a minimum, a side-chain substitution from a methyl to an isopropyl in the molecule should lead to a non-inhibition of the acetylcholinesterase. The variation of the administration of this so modified compound, e.g., a long-term infusion could increase the drug's uptake. To prove the selectivity towards brain tumors the drug has to be tested in rodents, bearing xenotransplanted glioblastomas or astrocytomas.

However, the great potential for selective and rapid compound syntheses for BNCT by the well elaborated metallocene chemistry for roughly 30 years should be noted. Hence, it should be possible to create custom-tailored metallocene compounds which meet the various BNCT requirements.

Table 2. Mean boron concentrations in mouse tissue obtained from ICP-AES and ICP-MS analysis. All concentrations are given in μg/g

Time of sacrifice [h]	Brain	Liver	Kidneys	Spleen	Lungs	Muscle
0.5	2.4 ± 0.6	24 ± 7	12 ± 2	37 ± 8	10 ± 2	6 ± 3
1.0	2.3 ± 0.5	19 ± 8	8 ± 1	32 ± 15	8 ± 2	7 ± 2
1.5	2.0 ± 0.6	17 ± 5	9 ± 3	28 ± 3	5 ± 1	5 ± 2
2.0	1.8 ± 0.6	20 ± 9	9 ± 3	22 ± 5	12 ± 4	5 ± 2
4.0	1.3 ± 0.7	19 ± 8	9 ± 3	19 ± 5	15 ± 3	7 ± 3

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