Biological Efficacy of Boronated Low-Density Lipoprotein for Boron Neutron Capture Therapy as Measured in Cell Culture


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ABSTRACT

Low-density lipoproteins (LDLs) are known to be internalized by the cell through receptor-mediated mechanisms. There is evidence that LDLs may be taken up avidly by tumor cells to provide cholesterol for the synthesis of cell membranes. Thus, the possibility exists that LDLs may provide an ideal vehicle for the transport of boron to tumor cells for boron neutron capture therapy. A boronated analogue of LDL has recently been synthesized for possible application in boron neutron capture therapy. The analogue was tested in cell culture for uptake and biological efficacy in the thermal neutron beam at the Brookhaven Medical Research Reactor. It was found that boron concentrations 10 times higher than that required in tumors for boron neutron capture therapy were easily obtained and that the amount of uptake was consistent with a receptor-mediated binding mechanism. The measured intracellular concentration of ~240 μg 10B/g cells is significantly higher than that obtained with any other boron compound previously evaluated for possible clinical application.

INTRODUCTION

BNCT is a binary system based on the interaction of thermal neutrons generated by an external beam, with 10B selectively localized in tumor cells. The high linear energy transfer products of the 10B(n,α)7Li reaction have a range of ~1 cell diameter, thus in principle permitting the selective exposure of cancer cells to radiation with optimal radiobiological characteristics. Previous clinical trials in the United States (1951–1961) were carried out with inorganic boron compounds showing no preferential uptake in tumor, with poor results. Since 1968, ~100 brain tumor patients have been treated in Japan with an improved (second-generation) compound, BSH. BSH shows transient binding, with a biological half-life on the order of hours and a tumor: blood concentration ratio of ~1.5 (1).

There is considerable interest in the initiation of clinical trials using BSH to treat brain tumors, in both the United States and Europe (2). However, we feel that the full potential of BNCT can be realized only through the utilization of third-generation compounds showing long-term intracellular binding to tumor (3–5). Such binding would permit clearance of normal tissue, and consequent tumor:normal tissue concentration ratios of 5:1 or 10:1, which are required to optimize therapeutic gain (5). Furthermore, an intracellular location is known to be ~10 times more effective than an extracellular or membrane-bound position (6). There are abundant data indicating that the distribution of BSH is predominantly extracellular (7–10).

The utilization of receptor-mediated uptake mechanisms (as are operational with LDLs) presents the possibility of long-term binding as well as internalization of the boron compounds. The metabolism of LDLs has recently been explored by Brown et al. (11) and Goldstein et al. (12, 13). LDLs, the major vehicles for transportation of cholesterol from plasma to cells, bind to receptors on mammalian cells and are subsequently internalized via receptor-mediated endocytosis and digested in the lysosomes, thus providing cholesterol for the synthesis of cell membranes. It has been estimated that up to 90% (or more) of cholesterol input is derived from receptor-mediated degradation of LDLs, and less than 10% is from cholesterol synthesized within the cell (14).

Selective targeting of boron via boronated LDL analogues is predicated on the assumption that LDL uptake is inherently higher in cancer cells. This follows on the assumption that cholesterol utilization will be greater due to increased cell membrane synthesis as a consequence of high turnover and replication rates. While LDL metabolism is a new field, with little overall knowledge of LDL receptor site densities in normal and transformed cells, there are a number of reports of significantly increased LDL uptake in cancer cells (14–18). For example, uptake rates were 3–10 times higher in blood mononuclear cells from patients with, as opposed to cells from patients without, acute myelogenous leukemia (14). Similar findings obtain for several gynecological malignancies where rates 50 times those in nonneoplastic gynecological tissues were found (15, 16). In vivo studies have also demonstrated rate of uptake of LDLs in a murine fibrosarcoma which was 2.5 to 10 times that found in liver, spleen, and muscle (18).

LDLs can be isolated, and the cholesterol ester core can be removed and replaced by a comparable hydrophobic material, which when reconstituted into LDLs can be a realistic analogue. This has been done by one of us (19) so that the resulting LDLs (~3 x 10⁸ daltons; protein content, ~6 x 10⁵ daltons), which are 20% boron/unit protein weight, have ~12,000 boron atoms/LDL (19). It is estimated that the receptor-bound LDL is internalized and delivered to the lysosome within 10 min (12) and that the rate at which LDLs enter the cell has a half-life of ~3 min at 37°C (20). Given an estimated receptor-site density of ~10,000 sites/cell (20), 10⁶ atoms of 10B could, in principle, be internalized into the cell in ~50 min. It is generally considered that at least ~10⁸ atoms/cell (~17 ppm 10B) are required for effective therapy in BNCT (3).

The data described here were obtained in an effort to demonstrate the biological efficacy of boronated LDLs based on the boron-thermal neutron reaction in vitro. Hamster V-79 and CHO cells were grown in the presence of boronated LDL for time periods of up to ~1 cell cycle (16 h). Following repeated washings in phosphate-buffered saline to remove unbound LDLs, cells were suspended in boron-free medium and irradiated at the thermal neutron beam of the Brookhaven Medical Research Reactor. Results showed that (a) boron concentra-
tions exceeding 10 times that needed for therapy were easily obtained; (d) boron remained firmly bound despite repeated washing and suspension in boron-free medium; (c) boron distribution was intracellular, with a biological efficacy indicative of a cytoplasmic location; (d) in vitro uptake and efficacy studies were consistent with a receptor-mediated binding mechanism; (e) boron uptake, retention, and efficacy were better by far than those obtained with any other boron compound previously studied in our in vitro system.

MATERIALS AND METHODS

Synthesis of Boronated LDLs. LDLs are spherical vesicles with a diameter of ~0.02 μm. Each LDL has a core of ~1500 long-chain fatty esters of cholesterol, surrounded by a polar coat of phospholipids, cholesterol, and one apoprotein (Apo B-100) with a mass of ~65,000 daltons. Human LDL was isolated from venous blood. Carburene carboxylic acid esters of fatty alcohols were used to replace the cholesterol ester core in the boronated analogue (19). It is assumed that the biochemical behavior of the LDL analogue is governed by the external shell and thus will not be altered by the boronated fatty alcohols used to replace the cholesterol core. In the following text the above analogue of LDL will be referred to as boronated low-density lipoprotein, or B-LDL.

Cell Culture Procedure. CHO and V-79 Chinese hamster cells in logarithmic growth were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (HyClone), 1% penicillin-streptomycin (Gibco), and 2.0 μM 1-glutamine (8, 10).

Cell Irradiation. Irradiations were carried out at the thermal neutron beam of the Brookhaven Medical Research Reactor in 1.5-ml Eppendorf microfuge tubes, with a thermal neutron fluence rate of 2.8 × 10¹⁰ neutrons cm⁻² min⁻¹ at the center of the tube (10, 21). Beam parameters are summarized in Table 1. γ-Irradiations were carried out using a 9000-Ci 137Cs source at the Biology Department's Controlled Environmental Radiation Facility, at a dose rate of ~0.5 Gy/min.

Iodination of B-LDL. For some experiments the boron-containing LDL was labeled with ¹²⁵I by the method described in (22).

Data Analysis. Survival was determined by colony assay; D₀ values were obtained by measuring the difference in irradiation time which reduced survival by a factor of 1/e on the linear portion of the curve. D₀ is expressed in units of time throughout this paper because the latter is the most basic parameter (10). Conversion to absorbed dose, biological effective dose rate, etc., can be carried out directly using the data in Table 1.

The efficacy of boronated LDLs was evaluated in terms of equivalent amounts of ¹⁰⁹B in the form of boric acid (H₃BO₃) or “boric acid equivalents” (10). This method of analysis is carried out to quantify the results of an unknown boron distribution, in terms of a known quantity such as boric acid, where a uniform distribution inside and outside the cell is assumed. To obtain BAEs, V-79 cells were grown in the presence of ~23 μg ¹⁰⁹B/ml of growth medium, washed thoroughly, pelleted, and adjusted to 1 g with deionized water prior to activation.

RESULTS

Biological Efficacy of Boronated LDLs. Hamster V-79 and CHO cells were incubated for ~1 cell cycle (16 h) at 37°C in the presence of ~23 μg ¹⁰⁹B/ml of growth medium, washed thoroughly (3 times in phosphate-buffered saline), and then irradiated in the thermal neutron beam. Results are shown for CHO and V-79 cells in Figs. 1 and 2, respectively. For comparison, survival curves obtained under similar conditions (30 μg ¹⁰⁹B/g) with BSH and a boronated porphyrin (10) are shown in Fig. 3. D₀ and the resulting boric acid equivalents for the

Table 1 Dose Rates for Cell Irradiations

<table>
<thead>
<tr>
<th>Component</th>
<th>cGy (min⁻¹)</th>
<th>cGy × RBE⁻¹ (min⁻¹)</th>
<th>% of total effective dose rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast neutrons</td>
<td>13</td>
<td>26</td>
<td>68</td>
</tr>
<tr>
<td>γ</td>
<td>6</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>³⁵N(n,p)³⁷C</td>
<td>3.1</td>
<td>6.2</td>
<td>62</td>
</tr>
<tr>
<td>Total</td>
<td>22.6</td>
<td>38.2</td>
<td>100</td>
</tr>
</tbody>
</table>

* An RBE of 2 is assumed for fast neutrons and the ³⁵N(n,p)³⁷C reaction (3).

Fig. 1. Survival curves for hamster CHO cells exposed to the thermal neutron beam at the Brookhaven Medical Research Reactor. Control cells were not exposed to drug. Test cells were grown for ~1 cell cycle in the presence of 23 μg ¹⁰⁹B/ml from boronated LDLs in the growth media.
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Fig. 2. Same parameters as in Fig. 1, except with hamster V-79 cells.

Fig. 3. Same parameters as in Fig. 2, but including survival curves from BSH and a boronated porphyrin for comparison.

above curves are summarized in Table 2. It can be seen that the efficacy of the B-LDLs is ~10 times greater than that of the boronated porphyrin, which itself is 10 times more effective than BSH.

To reduce the severity of response, cells were incubated in a reduced amount of B-LDL (~3.6 µg 10B/g growth medium). Absolute uptake was reduced by a factor of 2.6 as indicated in Tables 2 and 3, but the efficiency of uptake was increased (from 4.3 to ~10 µg 10B/g/µg administered) for the lower concentration of LDL (~5 µg 10B/ml growth medium; Table 3).

In an experiment concomitant to the high 10B exposures to CHO cells shown in Table 2 and Fig. 1, the average 10B content of cells was determined by neutron activation analysis of the prompt-γ from the 10B(n,α)7Li reaction. Analysis of CHO cells incubated for 1 cell cycle in 18.5 µg 10B/g of B-LDL and then washed 3 times prior to analysis showed an absolute concentration of 239 µg 10B/g. Since the biological efficacy is equivalent to ~100 µg 10B/g of boric acid (Table 2), one can infer that the boron location is predominantly intracellular. This follows from calculations of the effects of uniformly distributed boric acid in V-79 cells (6). Extracellular boric acid provides 10% of the dose to the nucleus, while cytoplasmic and nuclear boric acid provide 45% each. If, for example, the location of boron was extracellular, the absolute 10B concentration would have to be ~1000 µg/g to obtain the observed response. Thus these data are consistent with a primarily intracellular location (10).

Uptake of B-LDLs versus Duration of Incubation. Cells were incubated in ~5 µg 10B/ml growth medium, for time periods varying from 2 to 16 h, and then irradiated with thermal neutrons. Results, in terms of boric acid equivalents, are shown in Fig. 4 (and tabulated in Table 3). Uptake is seen to increase linearly during the exposure period, indicating a continuously operating active transport mechanism. In some experiments heparin was added to the final washing solution to release LDLs that were bound to receptors.

Uptake of B-LDLs at 4°C. Preliminary studies were carried out at 4°C, where the lower temperature was expected to decrease cellular metabolism, including receptor-mediated mechanisms. Although binding does occur at this temperature, internalization of the receptor-bound molecules is reduced (25). Uptake was reduced by a factor of ~7 as shown in Table 3, which is consistent with a metabolic mechanism reduced in efficiency by the lower temperature.

B-LDL Degradation, B Retention, and Site Density. In a further effort to evaluate uptake of B-LDLs, the latter were radiolabeled with 125I, to a specific activity of 12 × 10^6 dpm/mg protein, or 5.8 × 10^6 dpm/µg 10B (assuming 20% of protein weight = 10B). V-79 cell monolayers were incubated for 16 h at 37°C in the presence of 4.3 µg 10B/ml as evaluated by direct boron analyses (4.4 µg 10B/ml as evaluated by radioactive analysis of medium). Analysis of the cells after the growth period indicated 11.3 µg 10B/g cells, when evaluated according to the amount of radioactivity remaining in the cell sample. The boric...
Acid equivalent $^{10}$B concentration was 48 $\mu$g/g as determined by the aforementioned method, thus indicating an absolute $^{10}$B concentration of $\sim$100 $\mu$g $^{10}$B/g. Therefore the radioactivity was evidently no longer associated with $\sim$90% of the resident $^{10}$B. This indicates degradation of the LDLs and elimination of the iodine, with which the protein shell was labeled, with a concomitant intracellular retention of boron.

A similar experiment was carried out at 4°C. Cells were grown in the same growth media (4.3 $\mu$g $^{10}$B/g) and then evaluated for $^{10}$B incorporation by analysis of remaining radioactivity after a 2-h growth period. Results showed 4.8 $\mu$g/$^{10}$B/g. or $\sim$24,000 LDLs/cell or 1.1 $\mu$g $^{10}$B/unit administered; the latter is about 2 times that found in previous cold experiments in terms of boron acid equivalents. The higher boron content determined by analysis of radioactivity is attributed to the reduced biological efficacy associated with a cell membrane or cytoplasmic location (and therefore higher requisite $^{10}$B concentration) relative to a uniform boron distribution as assumed for boron acid equivalents. Furthermore, there was no indication of dehalogenation, suggesting that the loss of $^{125}$I label at 37°C was due to B-LDL degradation.

Sensitization of B-LDLs to Photons. A test was carried out to evaluate possible radiation-sensitizing effects of B-LDLs which are not a consequence of the $^{10}$B(n,0)$^{7}$Li reaction. CHO cells were grown for 14 h in the presence of $6\mu$g $^{10}$B/g from B-LDLs and then irradiated with $^{137}$Cs $\gamma$-rays at a dose rate of 0.5 Gy/min. Survival curves were identical to those obtained with untreated cells, indicating no effects from possible “conventional” radiation sensitization (data not shown).

**DISCUSSION**

Clearly mammalian cells require cholesterol for plasma membrane synthesis. While this may be derived either from exogenous sources or as a result of *de novo* synthesis, the bulk is preferentially obtained from receptor-mediated degradation of LDLs (14, 20). The latter follows in part because there is no known mechanism whereby large nonpolar molecules such as cholesterol esters can passively cross cell membranes. The LDL pathway provides a high-affinity receptor-mediated mechanism, such that LDLs can be internalized to meet the cell’s requirements for membrane biosynthesis while minimizing requisite plasma concentrations.

The cellular content of cholesterol is regulated by the number of LDL receptors, so that only enough cholesterol is taken up to satisfy requirements of new membrane synthesis and replacement of sterol due to membrane turnover. For example, fibroblasts grown continuously in the presence of LDLs express a receptor site density <10% of that present at full capacity (20). The rate of cholesterol synthesis is controlled by the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (26). Overaccumulation of cholesterol is prevented by a suppression of cholesterol synthesis through the reduction of enzyme activity, resulting from a decrease in the rate of enzyme molecule synthesis. Such a reduction is evidently caused by cholesterol delivered by receptor-induced endocytosis of LDL and not by cholesterol delivered by bulk phase (nonspecific) pinocytosis. The latter mechanism evidently does not significantly increase cholesterol content of the cell or regulate cholesterol synthesis.

In principle, the above mechanism would be ideal for the internal and irreversible delivery of boron to the cell, as outlined in the following paragraph. As indicated before, the high-affinity receptors would enable rapid uptake of boronated LDLs. Upon lysosomal hydrolysis of the LDL particles, it is unlikely that the boronated fatty alcohol esters would be incorporated in membrane synthesis. Thus the pool of boronated LDLs would not contribute to the usable accumulation of cholesterol and would therefore not suppress enzyme activity and/or receptor transport. If there were no biochemical process for the removal of the carborane carboxylic acid esters of fatty acid alcohols, they would accumulate, as is evidently indicated in Fig. 3. This argument, if valid, suggests that other suitably hydrophobic boron compounds, which efficiently replace the cholesterol ester core of LDL, would be similarly transported and accumulated by this nonsaturatable mechanism.

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**Table 3 Uptake of boronated LDLs in CHO cells**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Temperature (°C)</th>
<th>Duration of pulse (h)</th>
<th>Boron concentration in growth medium ($\mu$g $^{10}$B/g)</th>
<th>$D_0$ (min)</th>
<th>Boric acid equivalents ($\mu$g $^{10}$B/g)</th>
<th>Boric acid equivalents/$\mu$g administered ($\mu$g $^{10}$B/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>37</td>
<td>14.6</td>
<td>3.6</td>
<td>0.49</td>
<td>38</td>
<td>10.3</td>
</tr>
<tr>
<td>11</td>
<td>37</td>
<td>16.1</td>
<td>4.32</td>
<td>0.4</td>
<td>48</td>
<td>11.1</td>
</tr>
<tr>
<td>6</td>
<td>37</td>
<td>3.4</td>
<td>5.8</td>
<td>0.8</td>
<td>21</td>
<td>3.6</td>
</tr>
<tr>
<td>7</td>
<td>37</td>
<td>5.9</td>
<td>5.1</td>
<td>0.6</td>
<td>30</td>
<td>5.9</td>
</tr>
<tr>
<td>8</td>
<td>37</td>
<td>9.3</td>
<td>5.4</td>
<td>0.5</td>
<td>38</td>
<td>7.0</td>
</tr>
<tr>
<td>9</td>
<td>37</td>
<td>2.8</td>
<td>2.4</td>
<td>1.3</td>
<td>10.5</td>
<td>4.4</td>
</tr>
<tr>
<td>10*</td>
<td>37</td>
<td>4.75</td>
<td>4.9</td>
<td>0.7</td>
<td>24.8</td>
<td>5.0</td>
</tr>
<tr>
<td>11S</td>
<td>4</td>
<td>2</td>
<td>5.6</td>
<td>1.9</td>
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<td>0.93</td>
</tr>
<tr>
<td>12S*</td>
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<td>6.3</td>
<td>2.2</td>
<td>3.6</td>
<td>0.64</td>
</tr>
<tr>
<td>13S*</td>
<td>4</td>
<td>2</td>
<td>6.3</td>
<td>2.1</td>
<td>3.8</td>
<td>0.60</td>
</tr>
</tbody>
</table>

* Heparinized.

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**Fig. 4.** Response of hamster CHO cells, in terms of boron acid equivalents, which were exposed to thermal neutrons following various incubation times in growth media containing boronated LDLs. Results are expressed in terms of response (boric acid equivalents) per $\mu$g $^{10}$B/g of growth media. Data obtained from heparinized cells.
In these experiments, the fetal bovine serum used was not lipoprotein deficient but contained 16 mg/dl of LDL protein (16 μg LDL/ml of cell media). Presumably the LDL concentration in the serum is sufficient to allow uninhibited synthesis of new membrane for cell division, as well as the usual membrane turnover required for cells not actively dividing. At a 10B concentration of 3.5 μg/ml, the B-LDL concentration would be ~17.5 μg/ml of cell media, so that a total of 33.5 μg LDL/ml would be offered to the cells (both boronated and unmodified LDL). If the modified and unmodified LDLs compete equally, changing the B-LDL content (from 3.5 to 23 μg 10B/ml, or from 17.5 to 115 μg B-LDL/ml) would change the available mixture from 48% to 88% B-LDLs, so that the 10B uptake, assuming constant turnover of receptor sites, would increase by ~83%. Data in Table 2 show that while the efficiency of uptake dropped by a factor of 2.5 (in terms of BAEs/μg 10B administered), indicating effects of saturation of sites with B-LDLs, the absolute uptake of 10B increased by ~2 times as the 10B administered increased by 6.6 times. The above may indicate an increase in receptor-mediated uptake of LDLs in response to decreased availability of cholesterol. Data in Table 3 and Fig. 4 show no effects of cholesterol saturation as a function of time, at a 10B concentration of ~5 μg 10B/ml.

Comparison between the experiments where boronated LDLs were given for short intervals at two different temperature conditions (4°C and 37°C), where heparin was used in an effort to release LDL from its membrane receptor (Table 3), shows a significant increase in boron internalization at 37°C. This response is attributed to metabolically active receptor mechanisms at 37°C. These data mimic the results reported in (25).

For BNCT, where it is proposed to treat brain tumors such as glioblastoma multiforme, it is presumed that the blood-brain barrier will exclude boronated LDLs from normal brain tissue. For the latter application of BNCT, therefore, the mechanism of uptake (specific or nonspecific) of boronated LDLs may not be as important as the fact that intracellular uptake is high, with long-term binding. An intracellular location is desirable due to increased biological efficacy. Long-term binding is desirable, particularly in view of the 4 to 6 fractions proposed by an international advisory panel reviewing BNCT (2). Such binding will allow the superposition of distributions from multiple compound administrations, thus providing higher B concentrations and a more homogeneous distribution.

In the treatment of brain tumors, as well as with any application of BNCT, consideration must be given to the incorporation of B compounds in the endothelial cells forming blood vessels (in both normal and tumor tissue) in the treatment volume. These cells will not be protected directly by the blood-brain barrier but should benefit from a geometrical protective factor of ~3 stemming from the fact that cells external to the blood vessels should not contribute to endothelial cell dose (27). The question of endothelial cell uptake is related also to the general question in cancer therapy as to which cells should indeed be the target (i.e., should one attempt to attack the vasculature or the tumor cells directly?). With respect to B-LDLs, the general approach described above is predicated upon the exploitation of the biochemical pathway for LDLs, which is assumed to be more active in cells requiring cholesterol for membrane synthesis during cell division. Furthermore, it has been reported by Denekamp (28) that endothelial cells in tumor vasculature have a mitotic index ~20 times that of endothelial cells in normal tissues; she suggests that this pathway should be utilized to selectively attack the tumor vasculature. In principle, B-LDLs should be a candidate for such an approach, the efficacy of which, however, remains to be demonstrated in vivo.

From the data presented here, it is evident that if boronated LDLs can be effectively introduced in vivo, the possibility exists that extremely high levels of boron (~250 μg 10B/g) may be obtained (and retained) intracellularly in tumor cells. Such high levels should have major beneficial consequences for BNCT. Compounds now available and proposed for clinical applications rarely have concentrations in tumor exceeding 30 μg 10B/g and, in addition, excepting porphyrins, have transient B uptake, with biological half-lives on the order of hours.

REFERENCES


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