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Boron-Loaded Liposomes in the Treatment of Hepatic Metastases: Preliminary Investigation by Autoradiography Analysis

Franca Pavanetto, Paola Perugini, Ida Genta, Claudio Minoia, Anna Ronchi, Ubaldo Prati, Laura Roveda, Rosanna Nano[a]
[a] Department of Pharmaceutical Chemistry, University of Pavia, Italy
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Boron-Loaded Liposomes in the Treatment of Hepatic Metastases: Preliminary Investigation by Autoradiography Analysis

Franca Pavanetto, Paola Perugini, and Ida Genta
Department of Pharmaceutical Chemistry, University of Pavia, Italy

Claudio Minoia and Anna Ronchi
Laboratory of Environmental Hygiene and Industrial Toxicology, S. Maugeri Foundation, IRCCS, Pavia, Italy

Ubaldo Prati and Laura Roveda
Department of General Surgery, IRCCS Policlinic, S. Matteo, Italy

Rosanna Nano
Department of Animal Biology, Centre of Study for Histochemistry, University of Pavia, Italy

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Boronophenylalanine (BPA)-loaded conventional and stabilized liposomes were prepared by the reversed phase evaporation method to treat liver metastases by boron neutron capture therapy. Conventional vesicles were composed of phosphatidylcholine and cholesterol, molar ratio 1:1. To obtain stealth liposomes, GM_1 or PEG were included in the lipidic bilayer at a concentration of 6.67 or 5 mol%, respectively. Large unilamellar vesicles were formulated encapsulating BPA in the liposome aqueous compartment as a complex with fructose; BPA free base also was embedded into the lipidic bilayer. In vivo experiments were carried out after intravenous injection of liposome suspensions in BD-IX strain rats in which liver metastases had been induced. Alpha particle spectroscopy associated with histological analysis was performed to visualize boron spatial distribution in liver. Simultaneously, tissue boron concentrations were determined using inductively coupled plasma-mass spectroscopy. Results showed that PEG-modified liposomes accumulated boron in therapeutic concentrations (>30 μg boron/g tissue) in metastatic tissue. The PEG-liposomes could be further explored in enhancing boron delivery to tumor cells.

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Address correspondence to Paola Perugini, Viale Taramelli 12, 27100 Pavia, Italy. E-mail: pperugini@chifar.unipv.it

Keywords Antitumor Therapy, Boron Neutron Capture Therapy (BNCT), Boronophenylalanine, Liposomes

Chemotherapy is one of the main therapeutic approaches, besides surgery and radiotherapy, for the treatment of both primary and metastatic cancers. Since anticancer drugs are neither specific nor targeted to the cancer cells, the development of effective and innovative strategies for the selective transport of drugs to tumor tissues appears an urgent issue in the anticancer field.

Boron neutron capture therapy (BNCT) has been developed to selectively destroy tumor cells while sparing healthy tissues. This technique is based on the ability of α particles, produced by the irradiation of a ^{10}B compound with a beam of low energy neutrons, to destroy the cells in which boron is localized (Hawthorne 1993; Barth, Soloway, and Brugger 1996). To assure therapy effectiveness while preserving normal cells, the boron concentration in tumor tissue has to be greater than 20 μg ^{10}B/g and the ^{10}B concentration ratio between tumor and healthy tissue has to be at least 4 (Barth, Soloway, and Fairchild 1992). Several boron compounds such as mercaptoundehydrododecaborate, boron-containing amino acids, and boron-containing nucleosides have been studied for application in this therapy (Chen, Mehta, and Lu 1997).

In 1988, a group of surgeons and physicists developed the Taormina project to develop a novel BNCT application applied to liver metastases. Its basic concept is the thermal neutron treatment of the explanted liver, previously isolated and maintained

in an extracorporeal condition before reimplantation in the same donor patient—organ autotransplant (Pinelli et al. 1996).

Metastasis treatment points out the problem of tumor cell heterogeneity that is recognized as the principal cause of treatment failure in cancer, and it is a formidable obstacle to effective therapy and to selective targeting of antineoplastic agents to tumor tissue (Poste 1986). The development of drug delivery systems, such as liposomes as the boron compound vehicle, can be a good opportunity to enhance drug accumulation in tumors and to decrease healthy tissue exposure to α particle damage.

Although liposomes in general do not concentrate specifically in tumors, researchers have demonstrated that by varying size and lipid bilayer composition, it is possible to increase the liposome specificity for tumours (Uchiyama et al. 1995; Gabizon and Papahadjopoulos 1988); in fact, the inclusion of monosialoganglioside (GM_1) or such hydrophilic polymers as polyethyleneglycol (PEG) in the bilayer can prolong vesicle circulation time in the blood stream, avoiding uptake by reticuloendothelial system cells and increasing the extravasation through the leaky endothelium of tumor vasculature by passive convective transport phenomena. Moreover, liposome specificity permits the administration of relatively low doses of boron in comparison to other boron delivery modalities. Furthermore, vesicles can incorporate large amounts of hydrophilic boron compounds within the aqueous core and lipophilic species embedded in the lipidic bilayer (Hawthorne and Shelly 1997; Mehta, Lai, and Lu 1996).

The aim of this preliminary work was to evaluate the feasibility of using liposomes as a boron compound carrier intended for the application in the Taormina project. For this purpose, we formulated conventional and modified liposomes containing p-boronophenylalanine (BPA); the autoradiography technique was applied to in vivo studies to evaluate the distribution of boron compound between metastatic and healthy tissue. Usually, tissue boron analysis is performed by inductively coupled plasma-atomic mass spectroscopy (ICP-MS); the opportunity to support this technique with direct visualization of the samples by autoradiography could allow us to better distinguish boron distribution between normal and metastatic cells.

Boronophenylalanine, an amino acid analogue, is selectively taken up by active metabolic pathways in cells with elevated metabolism, such as tumor cells, especially in metastatic melanoma (Mallesch et al. 1994). Because of its low aqueous solubility, BPA is used in medical applications as a soluble complex with fructose (Mori et al. 1989). In our work, we chose boronophenylalanine for two reasons: first, it had been approved in clinical trials for the glioma and metastatic melanoma treatment; second, BPA can be encapsulated in liposomes both in the aqueous compartment as a complex with fructose and in the lipid bilayer as a free base solution.

Our investigation emphasizes the potential pharmacokinetic advantages of BPA-loaded liposomes in the administration of drug solution: lower injected dose and higher selective accumulation in tumor tissue.

MATERIALS AND METHODS

D,L,p-boronophenylalanine (BPA) (^{10}B enrichment $\geq 95\%$) was supplied by Boron Biologicals Raleigh, NC, USA. Egg L,α,phosphatidylcholine type XI-E (PC) 100 mg/ml chloroformic solution was supplied by Sigma Chemical of Milano, Italy. Cholesterol (Col), m.p. 147–150°, and D(-)fructose, $[\alpha]^{23}_{D} = -90.97°$ (0.15% w/v in phosphate buffer pH 8) were supplied by Carlo Erba, Milano, Italy. Monosialoganglioside (GM_1) was a gift of Fidia, Abano Terme, Italy. MPEG-DSPE (PEG) was supplied by Sygena AG, Liestal, Switzerland. All buffer solutions were prepared as indicated in the 1998 British Pharmacopoeia. All other reagents and solvents were of analytical grade.

BPA-Fructose Complex Characterization

UV Spectroscopy

Solutions at a constant concentration of BPA ($1.63 * 10^{-4}$ M) with different concentrations of fructose were prepared in phosphate buffer solutions at pH 8. The absorption spectra (230–400 nm) of these solutions were recorded using a spectrophotometer model DU7500 (Beckman, Furletton, CA, USA).

Optical Activity

Optical activities of BPA-fructose mixtures and boric acid-fructose mixtures in phosphate buffer solutions at pH 8 were determined at 20°C using a polarimeter model DIP-1000 (Jasco Corporation, Japan). Samples were prepared using a constant BPA or H_3BO_3 concentration and varying fructose amounts, as reported in Table 1. Because of the low solubility of BPA, the highest molar ratio obtainable for boronophenylalanine/fructose mixture was 1:1. Each sample was stored for 24 hr before analysis.

Liposome Preparation and Characterization

Unilamellar liposomes were prepared by a reverse-phase evaporation method previously described (Perugini and Pavanetto 1998); batches of liposomes were produced in triplicate and

TABLE 1
Fructose, BPA/fructose and H_3BO_3/fructose solutions prepared for optical activity analyses

Fructose concentration (% w/v)	BPA:fructose mol:mol	H_3BO_3:fructose mol:mol
5.40	1:20	1:7
4.00	1:13	1:6
2.00	1:7	1:5
1.50	1:6	1:4
1.00	1:5	1:3
0.50	1:4	1:2
0.40	1:3	1:1
0.30	1:2	2:1
0.15	1:1	4:1

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TABLE 2
Batches of boronophenylalanine-loaded liposomes (PC:Col 1:1 mol:mol)

Batch number	Membrane stabilizer conc. (mol%)	BPA solution
1	—	BPA-F
2	GM_1 (6.67%)	BPA-F
3	PEG (5%)	BPA-F
4	—	BPA_t
5	GM_1 (6.67%)	BPA_t
6	PEG (5%)	BPA_t

Each batch is produced in triplicate.
BPA-F = boronophenylalanine-fructose complex aqueous solution.
BPA_t = boronophenylalanine-fructose complex aqueous solution and boronophenylalanine metanolic solution.

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reported in Table 2. Conventional vesicles were prepared with PC:Col 1:1 molar ratio (batches 1 and 4); stabilized liposomes were obtained adding GM_1 or PEG into the lipid solution at concentrations of 6.67 mol% or 5 mol%, respectively (batches 2, 3, 5, and 6).

In batches 1, 2, and 3, boronophenylalanine was encapsulated only into the aqueous compartment as a complex with fructose (BPA-F) (8 mg BPA/ml); in batches 4, 5, and 6, the drug was encapsulated both into the aqueous compartment as BPA-F (8 mg BPA/ml) and into the lipidic bilayer by adding its methanolic solution (1 mg/ml) to lipid mixture in diethylether (BPA_t).

Briefly, the lipid mixture (60 mg) in chloroform:diethylether (1:1 v:v) solution was emulsified by sonication with phosphate buffer solution at pH 8 containing BPA-F to obtain the W/O (water/oil) emulsion. Liposomes were produced by reducing the emulsion to a gel in a rotary evaporator at 30°C, 150 rpm under vacuum for 1 hr. To obtain unilamellar liposomes, large vesicles were subjected to extrusion through a 100-nm pore size polycarbonate membrane, using an extruder device Liposo Fast Basic (Milsch Equipment, Laudenbach, Germany). The last step was to separate the free boron compound from vesicles by ultracentrifugation at 50,000 rpm, for 1 hr, at 4°C, in an ultracentrifuge model L7-65 with rotor type 60Ti (Beckman, Palo Alto, CA, USA).

Liposome morphology was detected by TEM analysis, after vesicle staining with a saturated acetyluranil aqueous solution, using a JEM 1200 EXII Electron Microscope (Jeol, Tokyo, Japan), at 80 kV. The particle size of liposomes was determined by using large-angle dynamic light scattering (Brookhaven, model BI-90, Holtsville, USA), to measure sizes between 0.01 and 1 μm. The results are the averages of three samples.

In Vivo Studies

Liver metastases were induced in BD-IX strain rats by intrasplenic inoculation of 20.10^6 DHDK12 TRb cancer cells (the line was established from colon carcinoma in BD-IX rats) as described previously (Pinelli et al. 1996). To obtain one portion of healthy hepatic tissue as a control in the same animal, one peripheral portal vessel was clamped during the cell injection. Splenectomy was then performed before the clamp removal.

Ten days later, boron-loaded liposomes (2 mg ^{10}B/Kg) were intravenously injected; after prefixed times blood samples were recovered and the animal was sacrificed, its liver extracted, washed by perfusion of glucose solution, and analyzed. To evaluate the lowest boron concentration suitable for autoradiography detection, 4 healthy animals were treated with liposome suspensions containing boron concentrations ranging between 0.5 and 2.5 mg ^{10}B/Kg.

Boron Analysis

Boron analysis of tissues and liposomal suspensions was performed by inductively coupled plasma-atomic mass spectroscopy (ICP-MS) using a Perkin Elmer Sciex, model Elan 5000 equipped with Coolflow CFT-75 Neslab, autosampler AS 90. Samples of ∼100 mg were mineralized with HNO_3 in a microwave oven; after suitable diluition with bidistilled water, samples were analyzed versus calibration curves made in water and in matrix. Calibration curve ranged between 1 to 10 ppb of ^{10}B. The detection limit for boron was 0.09 ppb.

Distribution of ^{10}B into Hepatic Tissue

To detect the boron distribution in hepatic tissue, samples of healthy and metastatic liver were frozen in liquid nitrogen and samples were sequentially cut, with a criostate Reichert-Jung (Vienna, Austria) in 10-μm sections, for histological analysis by hematoxilin-eosin technique, and in 50-μm sections for irradiation inside the thermal column of the nuclear reactor, Triga Mark II (General Atomic, San Diego, CA, USA), for 2 hr at 250 kW.

The α particles emitted from the irradiated ^{10}B traced on a neutronic radiography film; after irradiation, the film was developed in NaOH 2.5 N at 60°C for 20 min to visualize latent images (Altieri et al. 1989). The autoradiography images can be compared with histological analysis using a stereomicroscope Kombistereo Leica associated with a computer equipped with a program for image acquisition.

RESULTS AND DISCUSSION

Figure 1 shows ultraviolet (UV) spectra of BPA solutions with increasing concentrations of fructose. The maximum absorption of BPA is at 268 nm; with increasing fructose concentration, this absorption decreases. Since the absorption spectrum of BPA in UV region is due to the characteristic absorption of the benzene ring, the changes in the spectra with increasing fructose concentration are evidence of complex formation.

Optical activity analyses were performed to investigate which atoms of both compounds were involved in the BPA-fructose complex formation. Figure 2 shows the net optical activity, obtained by subtracting the fructose optical activity value from

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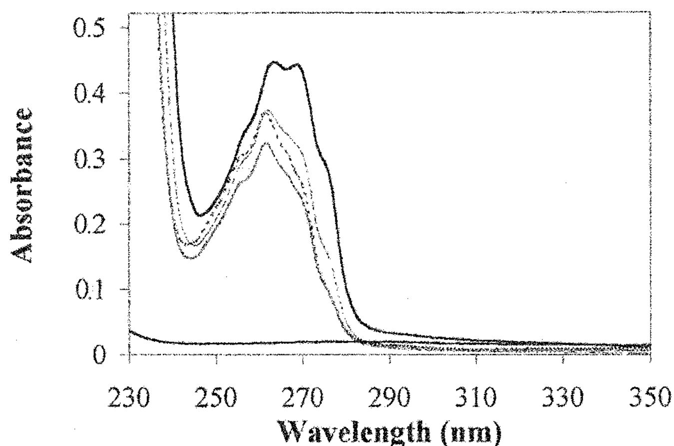


FIG. 1. UV spectra of BPA/fructose solutions compared with the UV spectrum of fructose (bottom line). Each spectrum from the top contains a concentration of fructose 0, 1, 2, and 3 times greater than that of BPA.

BPA/fructose or H_3BO_3/fructose mixture values, versus the mixture molar ratio. Optical activities of BPA-fructose complex solutions are different from those of fructose alone; since the BPA used is racemic, the changes in the optical activity of the complex involve a bond between the chiral atoms of fructose and the BPA.

Optical activity analyses of boric acid/fructose mixtures were carried out to understand if the boron in the BPA molecule is the atom involved in the link with the fructose. As shown in Figure 2, H_3BO_3/fructose mixtures present the same optical activity profile as BPA/fructose mixtures, confirming that the boron atom is involved in the complex formation.

Since the highest net optical activity obtained for both mixtures was related to the molar ratio 1:1, we can estimate that it is the stochiometric molar ratio in complex formation. This complex is used for preparation of liposomes by loading the hydrophilic boron compound into aqueous compartment.

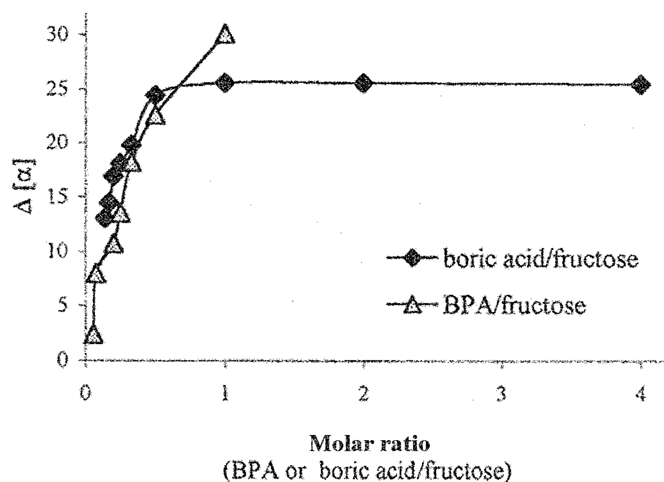
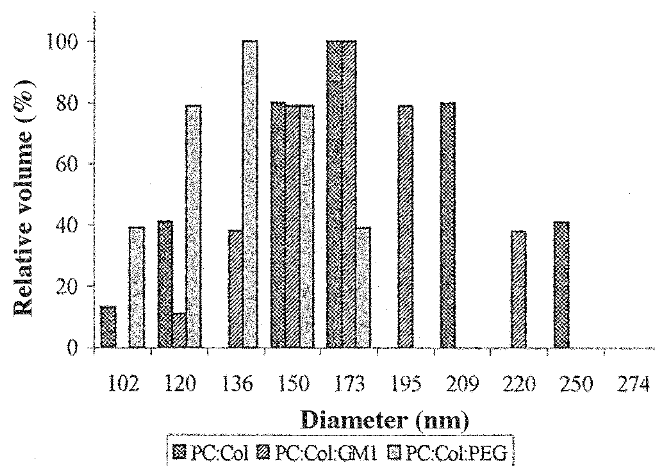


FIG. 2. Net optical activity of BPA/fructose solutions and of H_3BO_3/fructose solutions versus molar ratio.



Batch	d (nm) (s.e.)	I.P. (s.e.)
PC:Col	171 (4)	0.186 (0.027)
PC:Col:GM_1	195 (3)	0.209 (0.025)
PC:Col:PEG	156 (1)	0.153 (0.020)

I.P. polidispersity index; s.e. standard error

FIG. 3. Size analysis of BPA-F-loaded liposomes.

The REV (Reverse–Phase Evaporation) method associated with the extrusion process allowed us to obtain BPA-loaded liposomes well formed and with essentially a spherical shape and unilamellar structure, as confirmed by TEM analysis (data not reported). Particle size analyses by volume revealed a narrow size distribution for all types of liposomes produced. Differences between liposome batches are explained by different bilayer compositions, not the chemical form of BPA encapsulated; in fact, liposomes containing PEG have proven to be smallest both with BPA-F and with BPA_t. Figure 3 shows, as an example, size data concerning BPA-F-loaded liposomes.

Table 3 lists the encapsulation efficiencies of all liposome batches produced. The BPA content per lipid unit is always increased when a stabilizer agent (GM_1 or PEG) is added into the lipidic bilayer (batches 2 and 5; batches 3 and 6) relative to conventional vesicles (batches 1 and 4). The simultaneous use of both hydrophilic and lipophilic (BPA) chemical states led to a drug content per unit of lipid four times higher than liposomes carrying only BPA-F.

Table 4 lists the experiments successfully carried out in animals; in this preliminary work, the sacrifice times (T_S) at 3 and 6 hr were chosen to compare boron-loaded liposome distribution in tissues with previously reported data using BPA-F solutions (Pinelli et al. 1996). In this previous work, the authors demonstrated that the highest tumor-to-healthy tissue ^{10}B concentration ratios ($T > 4$) were achieved from 2 to 6 hr after boron solution injection.

The results of histological analysis performed by the morphological coloration technique are expressed as percentage of tumor area in the specimen, as shown in Table 4. Unfortunately,

TABLE 3
Theoretical and actual μmol BPA/μmol lipids and encapsulation efficiencies of all batches produced

Batch number	Liposome composition	Theoretical μmol BPA/μmol lipids	Actual μmol BPA/μmol lipids	Encapsulation efficiency (%)
1	PC:Col BPA-F	0.5	0.26	52.0
2	PC:Col:GM_1 BPA-F	0.5	0.32	64.0
3	PC:Col:PEG BPA-F	0.5	0.39	78.0
4	PC:Col BPA_t	2.0	1.13	56.5
5	PC:Col:GM_1 BPA_t	2.0	1.26	63.0
6	PC:Col:PEG BPA_t	2.0	1.64	82.0

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proliferation of metastatic tissue is very variable in animals, so many experiments failed because the tumor had not grown in the liver. Furthermore, the same pathological conditions could not be obtained for the different experiments.

Boron concentrations in blood, healthy tissue, and metastatic tissue, expressed as the percentage of injected dose, were obtained by ICP-MS analyses (Table 4).

Conventional liposomes show very low boron concentrations in blood and in liver at 3 hr from injection; this result agrees with earlier reports about the very rapid elimination of conventional liposomes by RES cells. These results are almost the same for both BPA chemical states encapsulated; this suggests that the BPA embedded in the lipidic bilayer does not modify in vivo behavior of liposomes, in terms of blood circulation time. Perhaps its effect continues inside the target organ, increasing selectivity for tumor cells (Chen et al. 1997).

The use of monosialoganglioside as a modifying agent did not seem to prolong the blood residence time of liposomes (Table 4); as pointed out previously by Liu (1996), GM_1 is not a good modifying agent to produce long-circulating liposomes in a rat model.

Liposomes composed of DSPE-PEG showed suitable boron concentrations in blood and in tissues both at 3 and at 6 hr from injection ($\mu g\,^{10}B/g$ tissue > 20 $\mu g/g$). High boron concentrations in blood reflects the PEG-liposomes capability to avoid RES cells. Unfortunately, boron analyses of healthy and metastatic tissues did not reveal a good boron concentration ratio between metastatic tissue and healthy tissue; probably because tissue samples for ICP-MS analysis were macroscopically cut from washed liver in pieces of ∼100 mg and the percentage of tumor area was less than 50%. Thus, it was impossible to exactly separate healthy from metastatic tissue.

To better understand boron spatial distribution in tissues, it is useful to directly compare the autoradiography films with the histological images produced by adjacent sections. In the autoradiography images, tissue areas appear darker the greater their boron concentration; for this reason, it is important that the boron concentration in the sample be high enough to produce traces in the film when the specimen is irradiated. Figure 4 shows liver autoradiography slides obtained by injection of PEG-liposomes containing different boron concentrations in 4 healthy animals and sacrificed at 3 hr after administration. The results suggest that injection of about 1 mg ^{10}B/Kg is the lowest dose able to be detected by this technique. However, boron distribution appears uniform in the specimen (Figure 4).

Figure 5 shows metastatic tissue slides of experiments carried out by injection of BPA_t encapsulated into conventional and PEG-stabilized liposomes: The animals treated with conventional

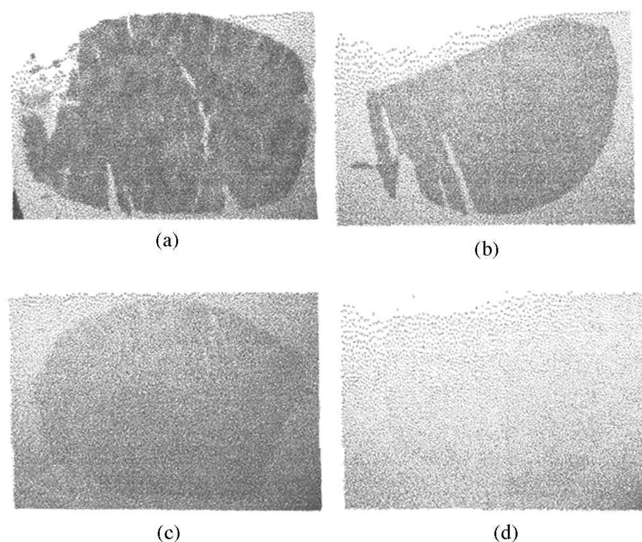

FIG. 4. Autoradiography slides of healthy liver tissues in rats sacrificed at 3 hr from injections of liposomes containing (a) 2 mg ^{10}B/Kg; (b) 1.5 mg ^{10}B/Kg; (c) 1 mg ^{10}B/Kg; (d) 0.5 mg ^{10}B/Kg.

TABLE 4
ICP-MS analyses of boron in blood, in healthy and metastatic tissues, sacrifice times (T_S), and histological reports at T_S of experiments successfully carried out in rats (inoculation of 2 mg ^{10}B/Kg in 2 ml liposome suspension)

Liposome composition	Sacrifice time (h)	Blood (%)	Healthy liver (%)	Metastatic liver (%)	Histological report (TT%)
PC:Col	3	0.22	1.14	0.8	15
BPA-F	6	0.4	1.89	1.49	10
PC:Col:GM_1	3	3.65	6.03	6.56	20
BPA-F	6	2.59	5.89	5.22	25
PC:Col	3	0.59	3.72	0.27	50
BPA_t					
PC:Col:PEG	3	26.97	34.48	14.26	35
BPA_t	6	20.05	27.83	21.77	50

TT% = percent of tumor tissue area on the sample; blood and healthy and metastatic tissue–boron concentrations are expressed as a percentage of injected dose.

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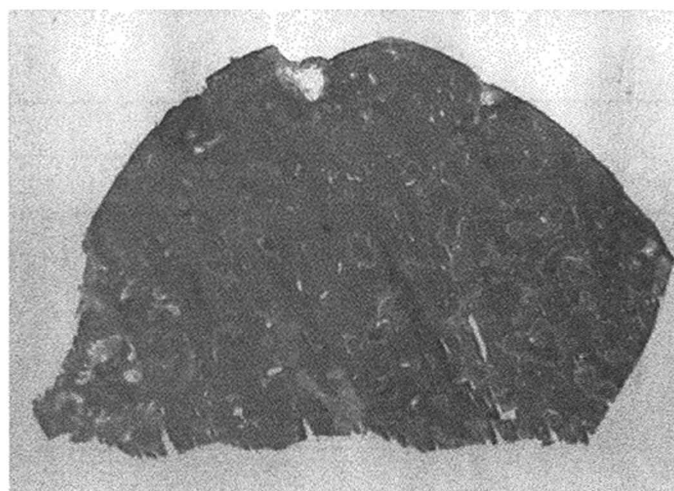
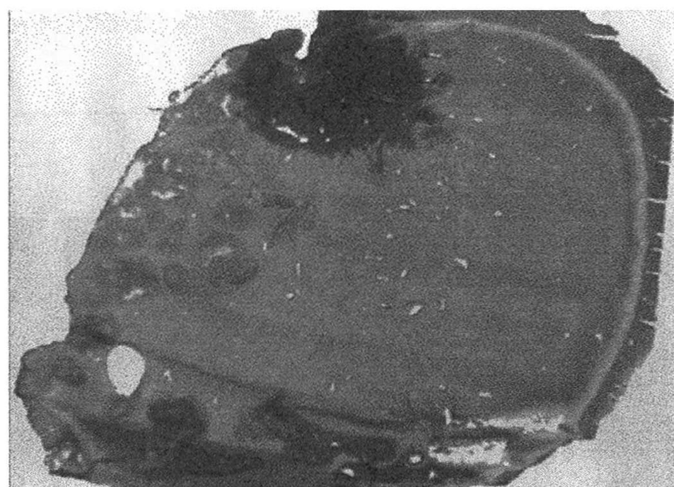
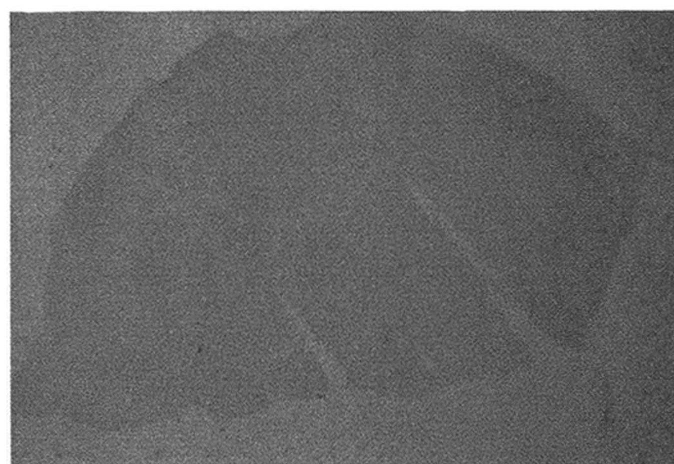


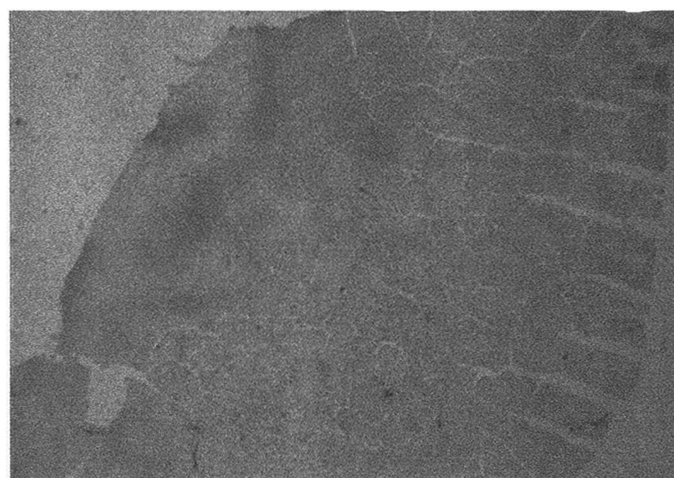
(a)



(c)



(b)



(d)

FIG. 5. Histological and radiography slides of metastatic tissue: (a) and (b) experiment using BPA_t-loaded conventional liposomes (sacrifice time 3 hr); (c) and (d) experiment using BPA_t-loaded PEG-stabilized liposomes (sacrifice time 6 hr).

liposomes were sacrificed at 3 hr from injection time while animals treated with PEG-stabilized liposomes were sacrified at 6 hr from injection.

Histological section micrographs permit one to distinguish healthy tissue, uniformly colored, from tumor areas that appear as well-defined spots (Figures 5a and 5c). The adjacent sections of the same liver were analyzed by autoradiography. BPA_t-loaded conventional liposomes did not reach the tumor area in a suitable concentration (Figure 5b); it seems that liposomes were already eliminated at 3 hr from injection; these data agree with conventional liposome characteristics, as described above.

The autoradiography slide of the experiment using PEG-stabilized liposomes shows the highest boron accumulation where the metastatic cells are (Figure 5d); this result is probably due to the PEG capability to prolong circulation time of liposomes in blood associated with BPA selectivity for tumor cells (Chen et al. 1997).

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SUMMARY

The results of our study suggest that unilamellar liposomes produced by reverse-phase evaporation method associated with the extrusion process are potentially useful boron delivery vehicles for application in BNCT. In our previous work, we evaluated the chemical and physical stability of liposomes carrying BPA; the results confirmed the use of liposomes as a carrier for boron compounds (Perugini and Pavanetto 1998). The structure of the liposome provides two modes of delivering boron-containing species in tumor tissue: Aqueous solutions of water-soluble boron salts can be concentrated into the internal compartment of vesicles, or lipophilic boron compounds can be embedded within the lipid membrane. The simultaneous use of hydrophilic and lipophilic boron compounds led to higher drug content and perhaps to a major selectivity for tumor cells due to BPA in the bilayer.

Results showed that liposome formulations containing PEG proved suitable for BNCT. In this preliminary work, we evaluated two different techniques for the boron concentration analysis into tissues; unfortunately, ICP-MS boron analysis cannot be effectively applied to metastatic tissue as previously related, and until now the autoradiography can be a qualitative analysis of boron spatial distribution. Further studies are being performed to quantify boron concentration directly from the autoradiography slides the α-particle spectroscopy; quantitative boron analysis is necessary to evaluate the tumor/healthy tissue boron concentration ratio. Obviously, it is also necessary to examine the pharmacokinetic parameters of BPA-loaded liposomes to assess BPA concentrations in blood and its tumor uptake to identify the most appropriate time for the treatment, which probably is $\sim$24 hr.

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