

Effect of O-4-ethoxyl-butyl-berbamine in combination with pegylated liposomal doxorubicin on advanced hepatoma in mice

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Abstract

AIM: To study the synergistic effects of calmodulin (CaM) antagonist O-4-ethoxyl-butyl-berbamine (EBB) and pegylated liposomal doxorubicin (PLD) on hepatoma-22 (H₂₂) *in vivo*.

METHODS: Hepatoma model was established in 50 Balb/c mice by inoculating H₂₂ cells (2.5×10⁶) subcutaneously into the right backs of the mice. These mice were divided into 5 groups, and treated with saline only, PLD only, doxorubicin (Dox) only, PLD plus EBB and Dox plus EBB, respectively. In the treatment groups, mice were given 5 intravenous of PLD or Dox on days 0, 3, 6, 9 and 12. The first dosage of PLD or Dox was 4.5 mg/kg, the other 4 injections was 1 mg/kg. EBB (5 mg/kg) was coadministered with PLD or Dox in the corresponding groups. The effect of drugs on the life spans of hepatoma-bearing mice and tumor response to the drugs were recorded. Dox levels in the hepatoma cells were measured by a fluorescence assay. Light microscopy was performed to determine the histopathological changes in the major organs of these tumor-bearing mice. The MTT method was used to analyze the effect of Dox or PLD alone, Dox in combination with EBB, or PLD in combination with EBB on the growth of H₂₂ cells in an *in vitro* experiment.

RESULTS: EBB (5 mg/kg) significantly augmented the antitumor activity of Dox or PLD, remarkably prolonged the median survival time. The median survival time was 18.2 d for control group, but 89.2 d for PLD+EBB group and 70.1 d for Dox+EBB group, respectively. However, Dox alone did not show any remarkable antitumor activity, and the median survival time was just 29.7 d. Addition of EBB to Dox or PLD significantly increased the level of Dox in H₂₂ cells *in vivo*. Moreover, EBB diminished liver toxicity of Dox and PLD. *In vitro*, EBB reduced the IC₅₀ value of Dox or PLD on H₂₂ cells from 0.050±0.006 mg/L and 0.054±0.004 mg/L to 0.012±0.002 mg/L and 0.013±0.002 mg/L, respectively (*P*<0.01).

CONCLUSION: EBB and liposomization could improve the therapeutic efficacy of Dox in liver cancer, while decreasing its liver toxicity.

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INTRODUCTION

O-4-ethoxyl-butyl-berbamine (EBB)^[1,2], a new derivative of bisbenzylisoquinoline, is one of the most powerful and specific calmodulin (CaM) antagonist with almost no cytotoxicity on normal cells. Its IC₅₀ value is 100 times lower than that of tetrandrine and in the same grade with R2457. Previous studies have shown that EBB has a strong inhibitory effect on the proliferation of hepatoma cells, and can prolong the life span of tumor-bearing mice. EBB augments the antitumor activity of 5-FU^[2], restores abnormal CaM content in major organs of tumor-bearing mice^[3] and improves their immunofunction^[4]. Therefore, EBB may have a synergistic effect with chemotherapeutic drugs and alleviate their organ toxicity clinically.

Doxorubicin (Dox) is a widely used anti-tumor agent. However, systemic treatment with Dox is complicated by its dose limiting toxicity, even at relatively low concentrations, as well as its rapid plasma clearance and distribution to non-relevant tissues^[5-10]. Pegylated liposomal doxorubicin (PLD) not only increases concentration of Dox in tumor and thus enhances its antitumor activity, but also has lower toxicity to the cardiac muscle compared with Dox alone^[7,11-15]. In this study, we adopted two strategies to enhance the anti-tumor activity and lower the cytotoxicity of Dox: Dox was administered in liposome form and in combination with EBB.

MATERIALS AND METHODS

Reagents

EBB was kindly provided by Dr. Xu YH (Institute of Molecular Biology, Nankai University, China). Hydrogenated egg phosphatidylcholine (HEPC) was kindly supplied by Lipod (Ludwigshaven, Germany). Polyethylene glycol-distearoyl phosphatidylethanolamine (PEG-DSPE) was purchased from Pharmachemie (Haarlem, the Netherlands). PLD (stabilized long circulating liposomes, Dox-HEPC-SLL) with an average diameter of 80 nm was prepared as described earlier^[16].

Animals and tumor model

Age- and sex-matched Balb/c mice (weighting 18-22 g) from the Animal Breeding Center of Peking University (Beijing, China) were used. H₂₂ cells in 0.2 mL (2.5×10⁶) were inoculated subcutaneously into the right backs of the mice. Tumor became apparent about 7 d after the inoculation, and the mice died approximately 18 d later without treatment.

Treatment protocol

On d 7 after inoculation, tumor-bearing mice were randomly divided into 5 groups. Control group received only saline. PLD or Dox group received 4.5 mg/kg PLD or Dox *i.v.* on the first

day, followed by 4 dosages of 1 mg/kg PLD or Dox in 3-d intervals. PLD+EBB or Dox+EBB group was treated in the same way, except that EBB (5 mg/kg, toxicity-free dosage)^[2] was coadministered. All these dosages could be well tolerated by mice (Dr. Yang SG, unpublished observations).

Assessment of tumor response

Tumor growth was recorded before and after the treatment by caliper measurements, and tumor size was calculated using the formula $0.4 \times (A^2 \times B)$ (where B represents the largest diameter and A the diameter perpendicular to B). Tumor response was also assessed by the life span of mice.

Tumor response rate was assessed on d 0 and 10 after commencement of the treatment as follows: progressive disease (PD) = increase in tumor size above 25%, no change (NC) = tumor size equal to that at the beginning of treatment (at a range of -25% and +25%), partial remission (PR) = decrease in tumor size between -25% and -90%, and complete remission (CR) = decrease in tumor size between -90% and -100%.

Drug levels in tumor tissue^[17]

Seven days after inoculation, Dox (10 mg/kg) or PLD (equal to 10 mg/kg Dox) alone, or each of them in combination with EBB (5 mg/kg) was injected *i.v.* After 1, 18, and 36 h, the mice were anesthetized with pentobarbital. Tumors were excised immediately after being perfused with saline. Tissues were homogenized and subjected to acidic isopropanol extraction, and Dox level was measured by a Perkin-Elmer Model MPF44 spectrofluorometer using an excitation wave at 490 nm and the emission wave at 590 nm. Fluorescence intensity was translated to μg or ng of Dox equivalents using a standard curve of Dox.

Histological examinations

One of the major objectives of these studies was to assess whether the treatment with Dox or PLD in combination with EBB would result in any significant alleviation of their tissue damages. Light microscopy was performed to determine the histological changes in organs from tumor-bearing mice treated with saline and drugs. Mice were sacrificed by cervical dislocation on d 20. The liver, spleen, kidney, lung, and heart were moved and fixed in formalin solution and cut into 4 μm thick sections. The tissue sections were hematoxylin-eosin stained and accessed by conventional histological criteria.

Determination of anti-tumor activity in vitro

H₂₂ cells were cultured at the concentration of 1×10^7 cells/L in 24-well plates in RPMI1640 culture medium containing 100 mL/L heat-inactivated fetal calf serum, 100 U penicillin, and 1 000 U streptomycin at 37 °C, 50 mL/L CO₂. Dox (0.01-0.20 mg/L) or PLD (0.01-0.20 mg/L) alone, Dox (0.01-0.20 mg/L) + EBB (1.17 mg/L, the IC₅₀)^[2], or PLD (0.01-0.20 mg/L) + EBB (1.17 mg/L, the IC₅₀) were added. Cells were harvested 72 h later, and 50 μL of MTT reagent was added to each well followed by 4 h incubation at room temperature. Absorbance was measured at 540 nm. Four replicate experiments were performed, and IC₅₀ values were calculated.

Statistical analysis

SPSS9.0 for Windows 98 statistic software was used for data analysis. A P -value less than 0.05 was considered statistically significant.

RESULTS

Antitumor activity of DOX or PLD in combination with EBB

Intravenous administration of 5 injections of Dox or PLD in

combination with EBB (5 mg/kg) strongly inhibited the growth of tumor, and resulted in tumor regression in some mice. The median survival time was 89.2 d in PLD+EBB group and 70.1 d in Dox+EBB group, respectively, significantly longer than that of control group (18.2 d) and Dox group (29.7 d) (Figure 1, Table 1).

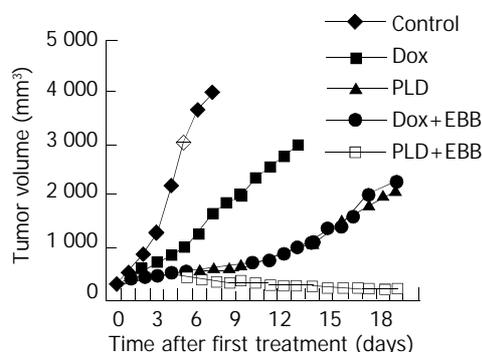


Figure 1 Antitumor activity of Dox or PLD alone or in combination with EBB in mice with hepatoma.

Table 1 Tumor response and survival time of hepatoma-bearing mice after systemic treatment with Dox or PLD alone or in combination with EBB

Group	Tumor response ¹					Mean survival time (d)
	PD	NC	PR	CR	(PR+CR)%	
Dox (n=10)	10				0	29.7±8.2 ^a
Dox+EBB (n=10)	5	2	3		30	70.1±8.3 ^{ac}
PLD (n=10)	6	2	2		20	67.2±9.1 ^{ac}
PLD+EBB (n=12)	2	1	6	3	75	89.2±13.2 ^{abg}
Controls (n=8)	8				0	18.2±2.7

¹Tumor response was scored on d 0 and d 10 after start of treatment. ^a $P < 0.05$ compared with control group; ^c $P < 0.05$ compared with Dox alone; ^e $P < 0.05$ compared with Dox+EBB; ^g $P < 0.05$ compared with PLD alone.

Drug concentrations in tumor tissues

The Dox levels in subcutaneous hepatoma were significantly increased by EBB in both Dox+EBB and PLD+EBB groups ($P < 0.01$) (Figure 2).

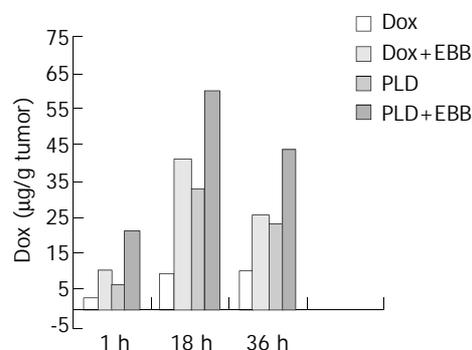


Figure 2 Dox concentrations in tumor tissues from mice after *i.v.* injection of Dox (10 mg/kg) or PLD (equal to 10 mg/kg Dox) in combination with EBB (5 mg/kg).

Histopathological changes

Severe histological changes were found in the liver of Dox group as compared to both Dox+EBB and PLD+EBB groups. Briefly, compared with control mice (Figure 3A), Dox-treated mice showed diffuse fatty degeneration and necrotic changes

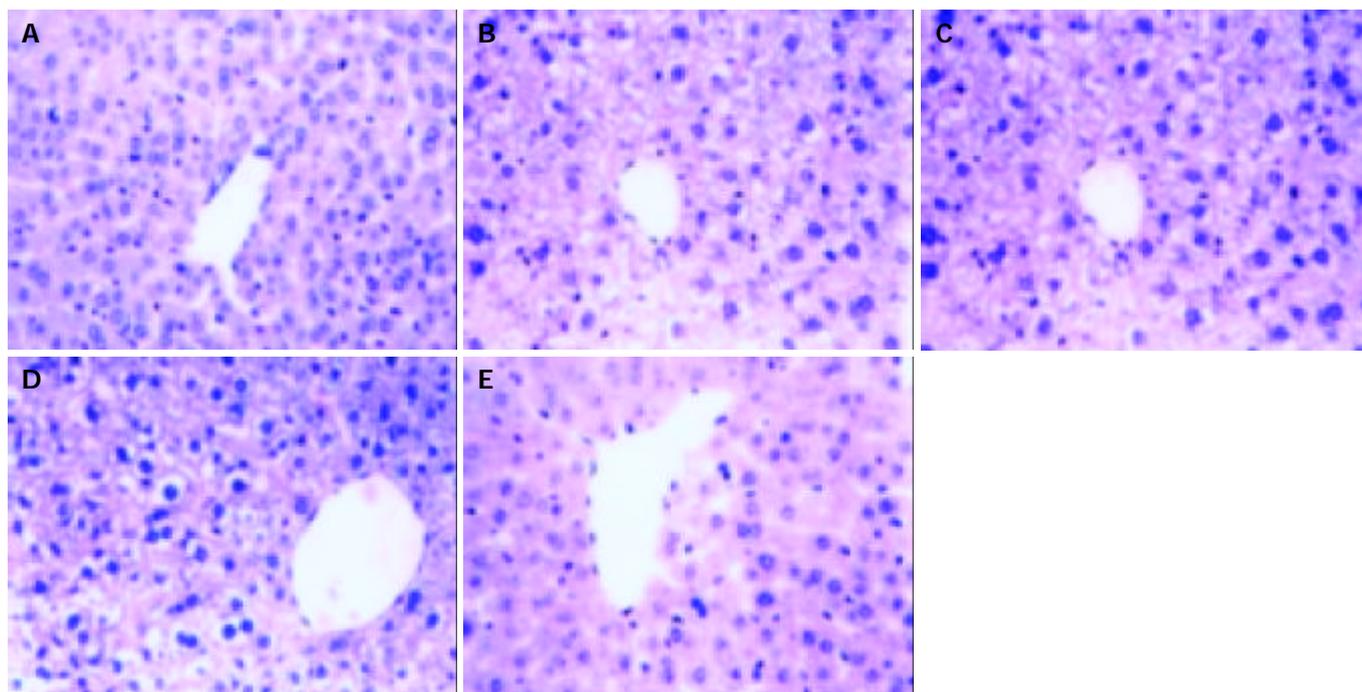


Figure 3 Histopathological changes in livers of tumor-bearing mice treated with saline (A), Dox (B), Dox+EBB (C), PLD (D) or PLD+EBB (E). (Original magnification, $\times 200$).

in the liver (Figure 3B). Livers from Dox+EBB-treated and PLD-treated mice showed similar change, but all were milder than Dox-treated mice (Figure 3C, D). Livers from PLD+EBB-treated mice showed very mild or even undetectable changes (Figure 3E). Severe histological damage was observed in the spleens from all drug-treated mice (data not shown). However, there were no significant abnormalities in the hearts, lungs, and kidneys from all animals (data not shown).

Synergetic effect of EBB with Dox or PLD *in vitro*

The *in vitro* experiment confirmed that EBB (1.17 mg/L, the IC_{50}) augmented the cytotoxicity of Dox and PLD, and reduced the IC_{50} of Dox or PLD on H₂₂ cells from 0.050 ± 0.006 mg/L and 0.054 ± 0.004 mg/L to 0.0120 ± 0.002 mg/L and 0.0130 ± 0.002 mg/L, respectively ($P < 0.01$, Figure 4).

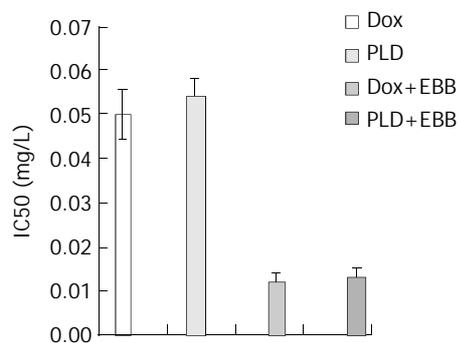


Figure 4 IC_{50} value of Dox or PLD alone or in combination with EBB.

DISCUSSION

Liposomes are attractive drug carriers for intravenous use because of their biocompatibility and versatility of formulation. As witnessed by publications, liposomes can be used for the delivery of cytotoxic drugs, antifungal agents, and biological response modifiers in humans. Phase I and some Phase II studies with liposomal doxorubicin have been reported. However,

the rapid and dominant uptake of these liposomes by the reticuloendothelial system affects its distribution in tumor^[18-20]. We have previously reported that encapsulation of Dox in long-circulating, pegylated liposomes could dramatically improve its mean residence time in serum^[16]. In the present study, we demonstrated the ability of sonicated liposomes to deliver DOX into H₂₂ cells. This may account for the increased antitumor effect of liposome-entrapped Dox observed in the model of hepatoma.

CaM is a ubiquitous calcium-binding protein that is responsible for many intracellular actions of calcium^[21-30]. Lot of evidences suggest that CaM not only plays an important role in the proliferation of normal cells, but also is related to hepatoma growth^[2,31]. In fact, an increased concentration of CaM has been demonstrated in malignant tissues and transformed cell lines^[32,33], and the correlation between inhibition of cell growth and antagonism of CaM has been observed^[21,34]. EBB, one of the strongest and most specific CaM antagonists with almost no cytotoxicity on normal cells, could decrease the amount of CaM in hepatoma cells and block the proliferation of hepatoma cells at G₂/M phase^[2].

In the present study, we demonstrated for the first time that EBB could strongly enhance the antitumor activity of liposomal doxorubicin. *In vitro*, EBB reduced the IC_{50} value of both Dox and PLD in inhibiting H₂₂ cells. *In vivo*, EBB enhanced accumulation of Dox in tumor tissue. There are at least three possible mechanisms underlying these effects. First, EBB could significantly enhance intracellular accumulation of Dox. Secondly, EBB could decrease the CaM content in cytoplasm, resulting in the inhibition of hepatoma cell growth^[2]. The third is that EBB upregulated the expression of wild-type p53 gene^[2], which is an antioncogene.

In terms of histological damage in the livers of the tumor-bearing mice, the present study showed that the changes were milder in mice treated with Dox+EBB and PLD+EBB than in mice treated with Dox or PLD alone. Unfortunately, severe histological damage was observed in the spleens of all experimental animals treated with drugs (data not shown). The reasons for these remain to be further investigated.

In conclusion, although Dox is extremely toxic when used

systemically, encapsulation of Dox in pegylated liposomes allows effective treatment of hepatoma when used in combination with EBB without potential hazards. In our model, administration of PLD resulted in a better tumor response compared with free Dox, and addition of EBB to PLD regimen resulted in a strongly enhanced synergistic tumor response. Therefore, in combination with EBB, the dosage of PLD can be reduced without loss of its antitumor activity, but with a decrease in cytotoxicity.

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