

FORMULATION OF BENDAMUSTINE HYDROCHLORIDE IN LONG CIRCULATING DPPC: CHOL LIPOSOMES, SURFACE MODIFIED WITH A PEO-BASED CO-POLYMER BEARING FOUR LIPID MIMETIC UNITS

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Summary: This study was aimed at determining the feasibility of DPPC:CHOL liposomes, sterically stabilized with a PEO-based polymer (DDGG)₄(EO)₁₁₄, previously validated as serum stable and long circulating formulation, to serve as a drug delivery platform for bendamustine hydrochloride. The drug loaded DPPC:CHOL:(DDGG)₄(EO)₁₁₄ LUVs were prepared by the lipid film hydration method, with successive freeze-thaw and extrusion cycles using an acidic solution of the drug in HBS (pH=2) as the dispersion medium. The liposomal formulation was characterized with average size of 153 nm, monomodal size distribution, and sustained release kinetics. The cytotoxicity evaluation has shown that liposomal bendamustine retains the biological activity of free drug and moreover after 4 day treatment is equieffective to it. These findings give us reason to consider DPPC:CHOL:(DDGG)₄(EO)₁₁₄ LUVs as a versatile drug delivery system for bendamustine hydrochloride.

Key words: bendamustine hydrochloride, sterically stabilized liposomes, targeted drug delivery, MTT-assay

ВКЛЮЧВАНЕ НА БЕНДАМУСТИН ХИДРОХЛОРИД В ПРОДЪЛЖИТЕЛНО ЦИРКУЛИРАЩИ DPPC:CHOL ЛИПОЗОМИ, ПОВЪРХНОСТНО МОДИФИЦИРАНИ С РЕО-БАЗИРАН КО-ПОЛИМЕР НОСЕЩ ЧЕТИРИ ЛИПИДО-МИМЕТИЧНИ ЗВЕНА

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Резюме: Настоящото проучване бе насочено към проучване на DPPC:CHOL липозоми, стерично стабилизирани с РЕО-съдържащия полимер (DDGG)₄(EO)₁₁₄ (валидирани като стабилен в серум и дългоциркулиращ състав) като лекарство доставяща система за bendamustine hydrochloride. Заредените с лекарственото вещество големи униламеларни липозоми на основата на DPPC:CHOL:(DDGG)₄(EO)₁₁₄ бяха приготвени чрез метода на хидриране на липиден филм, с последващи цикли на замразяване и размразяване и екструзия, като за хидриране на липидния филм бе използван разтвор на цитостатика в HBS (pH=2). Установено бе, че проучваният липозомен състав се характеризира със среден размер на везикулите 153 nm, мономодално разпределение по големина и забавено освобождаване на активното вещество. Проучването на биологичната активност показва, че липозомният bendamustine запазва фармакологичната активност на свободния цитостатик, и нещо повече, след 4-дневно третиране са еквивалентни. Тези данни ни дават основание да разглеждаме DPPC:CHOL:(DDGG)₄(EO)₁₁₄ липозомите като перспективна лекарство-доставяща система за bendamustine hydrochloride.

Ключови думи: бендамустин хидрохлорид, стерично стабилизирани липозоми, прицелно доставяне на лекарства, МТТ-тест

Introduction

Bendamustine hydrochloride is a nitrogen mustard alkylating agent, structurally related to chlorambucil, which has been elaborated in 1962 in the former German Democratic Republic, and since its very clinical introduction in 1969 has been used exclusively in this country up until the reunion of Germany [3, 6, 36]. The positive results from single-arm and controlled clinical trials that have been conducted thereafter fuelled the resurgence of this anticancer drug which is approved at present for the treatment of patients with chronic lymphocytic leukemia, rituximab-refractory indolent non-Hodgkin's lymphoma, and multiple myeloma [6, 10, 12-14, 19, 32, 37, 43, 44]. The drug has also demonstrated clinical activity in breast cancer [40, 42] and small-cell lung cancer [6].

Bendamustine hydrochloride is among the first rationally designed alkylating drugs, whose structure comprises three pharmacophore moieties: the bis-2-chloroethylamine alkylating group, a benzimidazole ring serving as a purine base mimic (suggesting possible antimetabolite effects), and a butyric acid side chain to increase water solubility [2, 11, 22]. Although its precise mode of action is not fully understood, bendamustine hydrochloride appears to act via distinct mechanisms compared to the classical alkylating agents in terms of DNA adduct formation, chemosensitivity and resistance patterns, cell cycle modulation, and recruitment of the apoptotic cell signaling pathways [2, 4, 11, 16, 19-22, 44].

Despite its emerging clinical role bendamustine hydrochloride has unfavorable physicochemical and pharmaceutical properties, translating into unfavorable pharmacokinetic behavior [3, 6, 33]. The drug is exclusively unstable in serum due to prompt hydrolysis yielding inactive hydroxy-derivatives (See Fig. 1), accounting for a very short 6-10 minutes half life of the first phase of the serum elimination curve [33, 45]. The rapid degradation of the drug in serum and the extensive liver metabolism impair its cytotoxic action within a short period of time, necessitating application of relatively high doses [3, 33]. This, in turn, is a prerequisite for increased exposure of non-target tissues and dose-limiting systemic toxicity [14, 38, 43]. Therefore bendamustine appears to be a good candidate for incorporation in nanoparticu-

late carriers, which in line with their well established propensity to alter the biodisposition of entrapped cargo [40, 41, 46] are expected to favorably tailor its pharmacokinetic properties. On these grounds different drug delivery systems for systemic application of bendamustine have been developed such as chemically immobilized polymer-drug conjugates [5, 31], β -cyclodextrin inclusion complexes [1], dendrimer carriers [39], and liposomes [7-9]. Owing to the outstanding biocompatibility, biodegradability, safety and biodisposition of the lipid-based vehicles [18, 40, 41, 46] the incorporation of bendamustine into long circulating liposomes might be a promising way to prolong its half life in plasma, and favorably alter its biodisposition. Up to now only a few attempts to incorporate bendamustine into liposomes are found in literature, which is due to the fast hydrolysis of the drug in aqueous milieu [7-9]. Two more recent studies have shown that incorporation of the drug into liposomes with acidic water compartment would hamper the hydrolysis process enough to allow bedside preparation of the final dispersion immediately before use [7, 8]. These formulations, however were composed of natural phospholipids, and were not surface modified to afford plasma stability and prolonged plasma circulation.

As far as anticancer drug delivery is concerned however, the liposomes should resist the interactions with serum opsonins, evade sequestration into the reticulo-endothelial system (RES) and display long circulation times [18, 40, 41]. Usually, such prolonged circulation time is attained by grafting the liposomal membrane with PEG-lipids, i.e. poly(ethylene glycol) modified phospholipids or other lipid-anchored polymers [18, 40, 41, 46]. Rangelov et al. have synthesized and characterized a series of PEO-based copolymers, which, in contrast to the conventional PEG-lipids, bear one to four blocks of lipid-mimetic anchors [35]. These have been found to ensure increased colloidal stability and/or prolonged circulation times of liposomes of different phospholipid composition [23-28, 34]. An analogue comprising an average of four lipid anchors $(DDGG)_4(EO)_{114}$ per molecule has been found to significantly prolong the plasma half-life and to hamper RES sequestration of DPPC:CHOL liposomes more efficiently than the commercial PEG-lipid DSPE-PEG-2000 [24].

In this paper we sought to determine the possibilities to utilize the validated serum stable and long circulating liposomal formulation DPPC:CHOL:(DDGG)₄(EO)₁₁₄ as a drug delivery platform for bendamustine hydrochloride.

Materials and methods

Materials

Dipalmitoyl phosphatidyl choline (DPPC), cholesterol (CHOL), phosphate buffer (pH 7.4), hepes buffered saline (pH 2.0), chloroform, RPMI 1640 growth medium, fetal calf serum (FCS) were obtained from Sigma Chemical Co (USA). (DDGG)₄(EO)₁₁₄ was synthesized, and purified as previously described [34]. Formic acid, 2-propanol, L-glutamine and ethidium bromide were purchased from AppliChem GmbH, Darmstadt, (Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Merck (Germany). Bendamustine hydrochloride was used as a commercially available sterile dosage form for clinical use. Sephadex G50 medium pre-equilibrated gel filtration columns were obtained from Pharmacia (Sweden).

Preparation of liposomes and physicochemical characterization of liposomes

Liposomes were prepared using the lipid film hydration method. DPPC, CHOL (2:1 molar ratio), 5 mol% (DDGG)₄(EO)₁₁₄ were placed into 50 ml round-bottom flasks and dissolved in chloroform. Thereafter the solvent was evaporated *in vacuo* using rotary vacuum evaporator (Buchi, Germany) until the formation of uniform lipid film. The flasks were placed in vacuum drier overnight in order to replace any traces of residual solvent. Bendamustine hydrochloride lyophilisate was dissolved in purified water and promptly diluted with equal amount of HEPES buffered saline (HBS) (pH 2.0; 2X solution) to yield a final concentration of the drug of 2 mg/ml. This acidic aqueous solution of bendamustine was used to hydrate the dry lipid film and the resulting dispersions were subjected to ten freeze-thaw cycles. Thereafter the liposomal dispersions were extruded five times through polycarbonate filters of pore size 200 nm and eight times through polycarbonate filters of pore size 100 nm using T001 thermobarrel extruder (Lipex Bi-

omembranes Ltd., Canada) to yield a homogenous population of large unilamellar vesicles (LUVs). The untrapped bendamustine was removed by gel filtration through a Sephadex G50 column (Pharmacia, Sweden), pre-equilibrated with phosphate buffered saline (PBS; pH 7.4).

The size and the size distribution of liposomes and their ξ -potential were determined on a NanoZS zetasizer apparatus (Malvern UK), equipped as a 4 mW He-Ne laser operating with polarized light of wavelength of 632.8 nm. The measurements were performed at 25 °C in PBS of pH 7.4.

Entrapment efficiency

Bendamustine hydrochloride loaded liposomes were analyzed immediately after preparation to determine the entrapment efficiency. Aliquots of the liposome dispersion were applied to Sephadex G50 gel filtration columns for removal of the untrapped bendamustine. In order to permeabilize the liposomes the aliquots were collected in graduated flasks and diluted with ethanol/methanol (90/10 v/v) in the ratio of 1:100. The samples were injected into the HPLC system for quantification of bendamustine hydrochloride, as previously described [31] with minor modifications. A Shimadzu LC – 10 Advp liquid chromatograph equipped with 4.6 mm x 250 mm column Tracel Excel RP-18, ODS with particle size 5 μ m and detector SPD 10 AVvp – UV-VIS with fixed analytical wavelength was used in the measurements. Mobile phase was acetonitrile: water: acetic acid (200:50:0.05 v/v/v), whereby the solvents used were filtered and degassed in advance. The quantification was carried out at 233 nm analytical wavelength, column temperature 25°C, and flow rate of ca. 1ml/min.

Bendamustine hydrochloride release kinetics

The dispersion was diluted with equal volume of PBS (pH 7.4) with or without 10 % fetal calf serum (FCS) and incubated at 37°C. At predetermined time intervals, was analyzed with respect to loss of intact incorporated drug. At the respective time-points aliquots of the aqueous solution were withdrawn from the release medium, purified from external drug by gel filtration and replaced with an equal volume of fresh medium to maintain sink conditions. The amount of

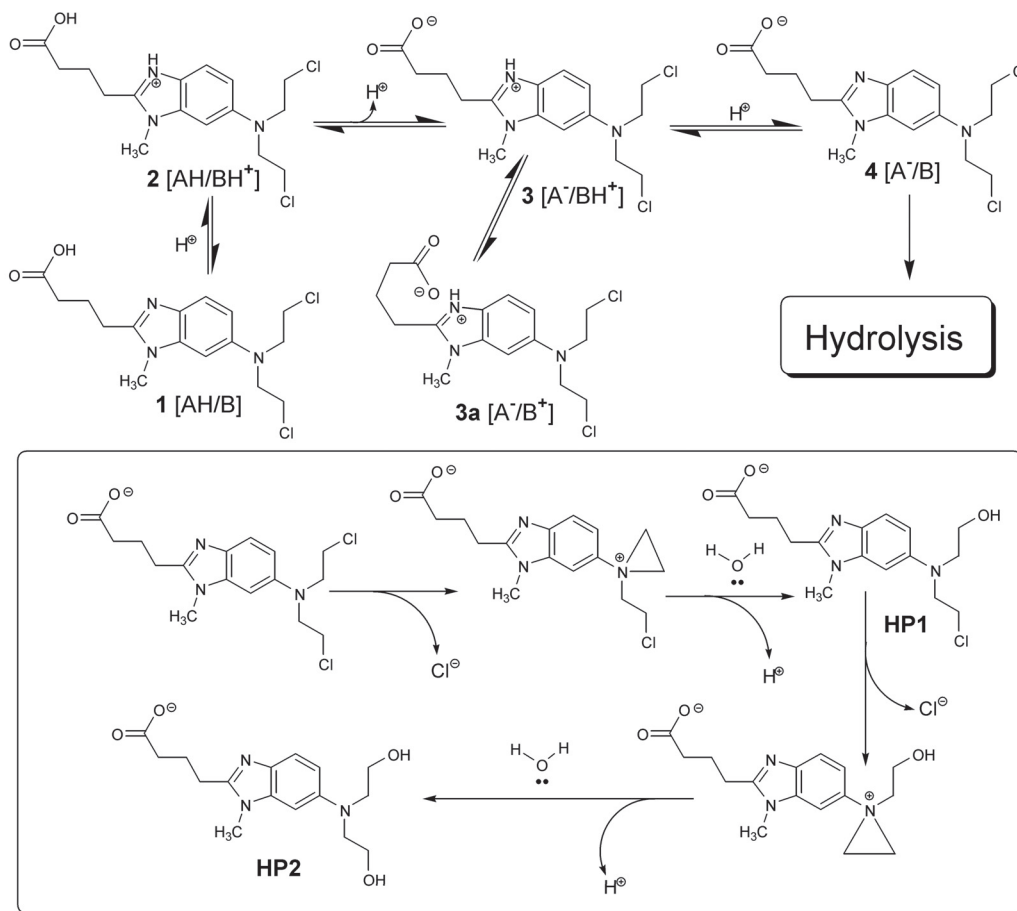


Fig. 1 Protolytic equilibria of bendamustine (1) in aqueous medium (leading to formation of acyclic (3) and pseudocyclic (3a) zwitterionic forms of the drug) and spontaneous hydrolysis (leading to formation of inactive mono- and dihydroxy derivatives - HP1 and HP2).

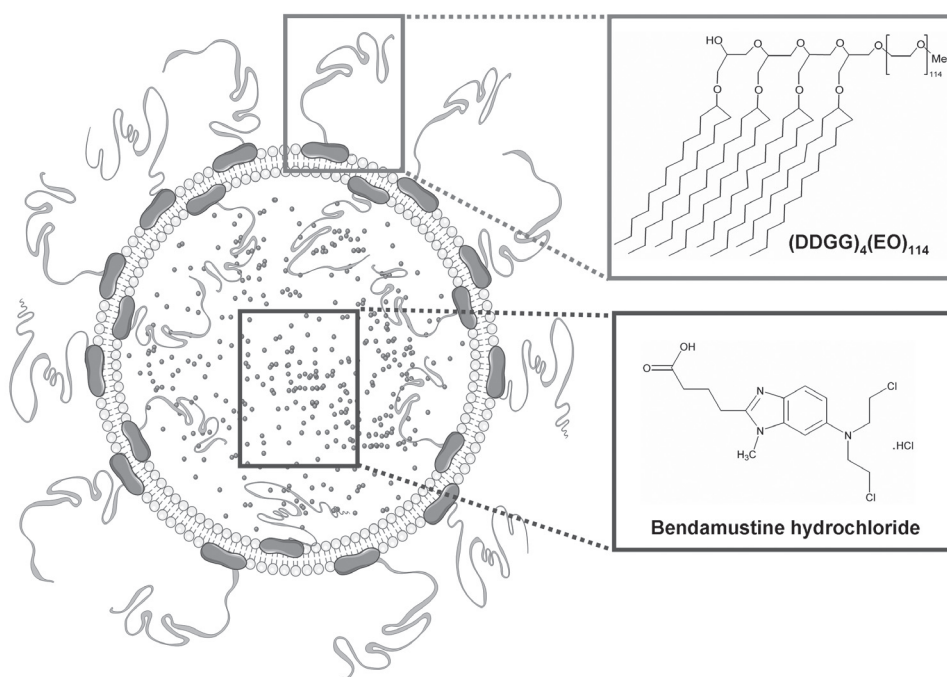


Fig. 2 Schematic representation of the DPPC:CHOL:(DDGG)₄(EO)₁₁₄ liposomal formulation as a drug delivery platform for bendamustine hydrochloride

bendamustine retained inside liposomes in each time interval was determined by HPLC using the procedure described elsewhere in the text.

Cells, culture conditions and cytotoxicity determination

SKW-3 cells (T-cell leukemia) were purchased from DSMZ GmbH, Braunschweig, Germany). The cells were grown in controlled environment – cell culture flasks at 37°C in an incubator, BB 16-Function Line[®] Heraeus (Kendro, Hanau, Germany) with humidified atmosphere and 5% CO₂. Cells were kept in *log* phase by supplementation with fresh medium, two or three times a week. The cytotoxicity of free vs. liposomal bendamustine hydrochloride was assessed by the 3-(4,5-dimethylthiazol2-yl)-2,5 diphenyltetrazolium bromide (MTT)-dye reduction assay to estimate cellular viability. Treatment was carried out for 48, 72 or 96 h. The procedure was carried out as described by Mosmann [29] with minor modifications [15].

The MTT data were normalized as percentage of the untreated control (set as 100%) and fitted to sigmoidal concentration–response curves and the corresponding IC₅₀ values were calculated using non-linear regression analysis (GraphPad Prism software package). Statistical processing exploited t-test and f-test with $p \leq 0.05$ set as significance level (GraphPad Prism software package).

Results and discussion

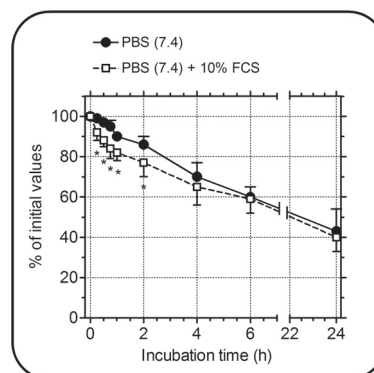
Bendamustine loaded DPPC:CHOL liposomes grafted with (DDGG)₄(EO)₁₁₄ (see schematic representation on Fig. 2) were prepared by the lipid film hydration method, using an acidic solution of the drug in HBS (pH=2) as hydrating medium and varying total lipid concentrations in the primary dispersions (ranging 5-20 μmol/ml). Optimal entrapment efficiency (34%) was encountered at a total lipid content of 20 μmol/ml, and all subsequent studies were carried out with this formulation.

The prepared liposomal dispersion had an average particle size of 153±4 nm and monomodal size distribution pattern (polydispersity index 0.07). The ζ-potential of the dispersions was – 9 mV. The established physicochemical and colloidal prop-

erties of the tested formulation are in agreement with those previously described for the non-loaded DPPC:CHOL:(DDGG)₄(EO)₁₁₄ carrier system [24], and seem to be unaffected by drug encapsulation and the acidic environment in the aqueous compartment.

Drug efflux experiments were carried out with PBS (pH 7.4) as the external dispersion medium. In order to imitate the *in vivo* conditions these were carried out at 37°C and moreover a parallel study was conducted with the presence of 10% FCS to evaluate the possible influence of serum opsonins and LDL on the membrane integrity. The drug efflux profiles from these studies are summarized in figure 3 and clearly indicate that the system ensures exceptional stability of the entrapped drug and its sustained release from liposomes.

Fig. 3 Efflux kinetics of bendamustine from the DPPC:CHOL liposomal formulation upon incubation in PBS pH 7.4, with or without the addition of 10% FCS, at 37°C over time (* indicates significant difference vs. incubation without FCS at $p < 0.005$; t-test).



The choice of the intrinsic pH was based on a comprehensive preformulation, and biopharmaceutical studies of bendamustine-loaded plain egg yolk phosphatidyl choline (EPC):CHOL liposomes, performed by Tove Julie Evjen from the University of Tromsø, Norway [8]. Albeit prone to very fast hydrolysis at physiological conditions bendamustine solutions appear to be fairly stable at pH 2, especially in the presence of relatively high sodium concentrations [8, 31]. As shown by Evjen additional benefit from the acidic internal compartment of liposomes is that it shifts the protolysis equilibria of bendamustine to the lipid bilayer impermeable AH/BH⁺ form (Fig. 1). At pH values ranging 4.5-6.3

(the respective pKa values) the drug exists predominantly in zwitterionic form A-/BH⁺ which being electroneutral is capable of crossing phospholipid membranes [8], that would ultimately lead to prompt release from liposomes and loss of their capacity to serve as drug reservoir systems. As prove of the principle the EPC:CHOL formulation showed excellent stability at room temperature and acidic incubation medium, but proved to lose its sustained release properties at physiologically relevant temperature and pH [8]. In contrast to these findings our formulation showed excellent stability and sustained release of the cargo at 37°C and pH 7.4, even in the presence of FCS and its bilayer destabilizing components. This discrepancy could be greatly ascribed to the formulation peculiarities, such as the surface modification and the bilayer composition. Thus we used a semisynthetic phospholipid with quite higher phase transition temperature (41°C) as compared to that of the natural extract EPC (ca. -6°C) [17], which enables DPPC:CHOL bilayers to be less leaky at physiological temperature and to resist opsonization [30]. These beneficial proper-

ties are further augmented by the steric stabilization with (DDGG)₄(EO)₁₁₄ which increases the colloidal stability of DPPC:CHOL LUVs and hampers the deleterious influence of serum components such as opsonins and LDL particles [24].

The cytotoxicity of free and liposomal bendamustine was tested in the T-cell leukemia SKW-3 after 48, 72 or 96 h exposure (Fig. 4; Table 1). At the shortest treatment period the results showed a marked shift of the concentration-response curve of liposomal drug to higher concentration as compared to the free drug. Such behavior of liposomal drugs is not unexpected considering the reservoir function of lipid vesicles and the sustained manner of drug release from them. At prolonged exposures however the difference between the dose-response curves and the IC₅₀ values between free and liposomal bendamustine were far less eminent and these actually did not differ statistically after the 96 h exposure.

These findings would be attributed to the ability of DPPC:CHOL LUVs to protect encapsulated drug against hydrolytic inactivation due to the high sodium content and low pH of their interior. In contrast

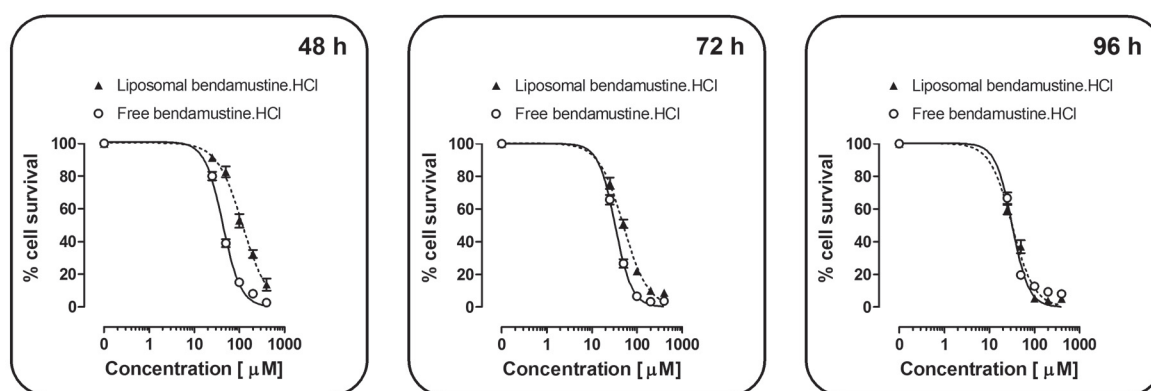


Fig. 4 Inhibitory concentration response curves of free and liposomal bendamustine hydrochloride against SKW-3 cells after 48, 72 or 96 h exposure (MTT-dye reduction assay).

Table 1. Cytotoxic effects of free and liposomal bendamustine hydrochloride against SKW-3 cells after 48, 72 or 96 h exposure (MTT-dye reduction assay)

Treatment series	IC ₅₀ (μM)		
	48 h	72 h	96 h
Free bendamustine. HCl	42.96 ± 1.04	32.76 ± 1.03	31.62 ± 1.72
Liposomal bendamustine. HCl	117.30 ± 7.10 ^a	49.83 ± 1.05 ^b	32.87 ± 2.08 ^c

^astatistically significant vs. free bendamustine. HCL ($p < 0.0001$);

^bstatistically significant vs. free bendamustine. HCL ($p < 0.0001$)

^cnon-significant vs. free bendamustine. HCL ($p = 0.731$) (f-test, GraphPad Prizm 4.0 Software for Windows).

the free drug is expected to promptly disappear due to degradation in the growth medium – the $t_{1/2}$ of the hydrolysis reaction at physiologically relevant pH and temperature is reportedly 10-14 minutes [8, 31].

Hence the *in vitro* cytotoxicity of free bendamustine is presumably due to a minor fraction which is capable of entering cell shortly after treatment, while the remaining drug would be either promptly and irreversibly bound to the serum proteins (abundant in the growth medium) or hydrolyzed to the inactive HP1 and HP2 products (Fig. 1). In a dissimilar fashion the liposomal pool provides an micro-environment whereby the drug is hydrolytically stable, protected from concurrent interactions with serum proteins or reactive nucleophiles abundant in the growth medium, and wherefrom it is continuously released. Thus albeit the initial exposure of tumor cells is significantly higher after treatment with the free drug the cumulative exposure in case of liposomal bendamustine is superior as clearly indicated by the significant increase of the cytotoxic efficiency with the prolonged dosing.

Conclusions

Bendamustine hydrochloride loaded DPPC:CHOL:(DDGG)₄(EO)₁₁₄ LUVs were prepared by the lipid film hydration method, using an acidic solution of the drug in HBS (pH=2). The liposomal formulation was characterized with average size of 153±4 nm, monomodal size distribution, 34% entrapment efficiency, and sustained release kinetics. The pharmacological study has shown that liposomal bendamustine retains the biological activity of free drug and moreover after 4 day treatment is equieffective. These findings together with the established benefits of prolonging the plasma circulation and mean retention time, and modulating the biodistribution of the drug upon encapsulation distinguish the DPPC:CHOL:(DDGG)₄(EO)₁₁₄ LUVs as a versatile drug delivery platform for bendamustine hydrochloride.

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