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Pharmacokinetic behavior and antineoplastic activity of liposomal hexadecylphosphocholine

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Abstract. Hexadecylphosphocholine (HePC) shows remarkable antineoplastic efficacy in Sprague-Dawley rats bearing methylnitrosourea-induced mammary carcinoma. Unfortunately, this is accompanied by detrimental side effects that include gastrointestinal damage, body weight loss, and thrombophlebitis after i.v. injection, which has precluded the use of the HePC in humans, where nausea and vomiting can occur at noneffective dose levels. We have developed small unilamellar vesicles (SUVs) composed of HePC, cholesterol, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol, which can be given p. o. and i.v. In contrast to the free drug, the toxicity of liposomal HePC is shown to be greatly reduced, and there is no risk of thrombophlebitis. Single administration of equimolar HePC doses results in differing pharmacokinetic values for free HePC (p. o.) and HePC-SUVs (p. o., i.v.).

Key words: Liposomes – Alkylphosphocholine – Pharmacokinetics

Introduction

Although the chemical structure and physical properties of hexadecylphosphocholine (HePC) are closely related to those of lysolecithins, the biological activities of the two differ greatly [15, 25]. In contrast to lysolecithins, HePC accumulates in tumors and other tissues [26, 28], and the absence of glycerol in its structure produces a variety of remarkable biological effects [7, 37, 40, 48], including reduced metabolism [16, 17]. Complete remissions of tumors chemically induced by treatment with methylnitrosourea (MNU) and dimethylbenz(a)anthracene (DMBA) in rats have been shown following oral treatment with HePC [4–6, 14, 18, 32, 33, 39], in contrast to their resistance to conventional chemotherapy [3]. Because multiple i.v. injection produces thrombophlebitis [30], oral administration is preferred. HePC is almost completely resorbed in the gastrointestinal tract [28].

In the late 1980s, HePC was introduced into clinical trials [44], and topical treatment was found to be successful in breast cancer patients with skin metastases without side effects [42, 43, 45, 47]. Therefore, HePC was approved by the German Bundesgesundheitsamt in 1992 for topical treatment and represents the first medicament to be based on a phospholipid-like structure.

In contrast, systemic treatment of patients has been found to produce side effects of nausea and vomiting at noneffective doses [41]. We now have developed a stable liposomal dispersion [21, 22] consisting of HePC, cholesterol (Chol), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (PPG3PG) that overcomes gastrointestinal toxicity and can be given by i.v. injection. The physicochemical stability, hemolytic behavior, and uptake of liposomal HePC in cultured tumor cells have previously been described [23]. This report deals with the reduction in gastrointestinal toxicity, the pharmacokinetic behavior, and the therapeutic efficacy of liposomal HePC following i.v. application in rats bearing MNU-induced mammary carcinoma.

Materials and methods

Chemicals. HePC was prepared according to Eibl and Engel [13] and was supplied by Asta Medica (Frankfurt, Germany). Chol was ob-

Abbreviations *AUC* area under the curve · *BWC* body weight change · c_{last} last measured concentration · c_{max} peak concentration · Cl_{tot} total body clearance · *Chol* cholesterol · *DMBA* dimethylbenz(a)anthracene · *HVD* half-value duration · *HePC* hexadecylphosphocholine · *HPLC* high-performance liquid chromatography · *HPTLC* high-performance thin-layer chromatography · *k* elimination constant · *MNU* methylnitrosourea · *PPG3PG* 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol · *5-D* Sprague-Dawley · *SUV* small unilamellar vesicle · t_a distribution half-life · t_B terminal elimination half-life · $t_{1/2}$ half-life · t_{max} peak time · *T/C* quotient of median tumor volumes of treated and control groups · V_d relative volume of distribution

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tained from Fluka Chemie AG (Buchs, Switzerland), and PPG3PG was supplied by Sygena (Liesthal, Switzerland). Solvents were purchased from Baker Chemicals (Deventer, The Netherlands).

Liposomes

The liposomal molar lipid ratio was 4:5:1 for HePC/Chol/PPG3PG. Small unilamellar vesicles (SUVs) were prepared in 154 m*M* NaCl (pH 6.0–7.0) using the French pressure cell [2]. HePC-SUVs were separated by centrifugation at 20,000 *g* (J2-21M/E; Beckman Instruments GmbH, München, Germany), sterile-filtered, and stored at 4°–8° C under nitrogen. SUVs showed a mean diameter of 70 ± 30 nm as determined by dynamic light-scattering analysis with a Nicomp C370 particle sizer (Pacific Scientific, Silver Spring, USA) [21]. The chemical stability and the molar ratio of the lipid compounds before and after preparation of the liposomes as well as after storage under different conditions were examined by high-performance thin-layer chromatography (HPTLC) as described by Rustenbeck and Lenzen [35] with modifications as described by Kötting et al. [27].

Animals. Female Wistar rats and female Sprague-Dawley (S-D) rats (age, 40 ± 1 days) were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Germany). They were kept under conventional controlled conditions (temperature, 22° ± 2° C; relative humidity, 55% ± 10%; dark-light rhythm, 12 h). Altromin pellets and tap water were given ad libitum. The body weight change (BWC) was determined as the mean weight change expressed as a percentage of the initial body weight for each group.

Treatment with HePC. Oral administration of free and liposomal HePC was carried out by gastric intubation once a week at weekly doses of 70, 100, or 150 mg/kg. An oral pulse dose of 150 mg/kg given once a week was tolerated better than an equimolar dose delivered five times a week [6, 18]. Injections of HePC-SUVs were given i. v. twice a week into the tail veins at weekly doses of 80 or 160 mg/kg HePC.

Analysis of HePC in serum. At various time points, blood was obtained by cardiac puncture from animals under anesthesia. After being centrifuged, the serum samples were frozen until the time of analysis. The HePC concentration was determined by HPTLC as previously described [27]. Total lipid extraction with $CHCl_3/CH_3OH$ (2:1, v/v) was repeated twice. The combined organic extracts were dried under nitrogen. Lipids were dissolved in $CHCl_3/CH_3OH/H_2O$ (30:60:8, by vol.) and applied to HPTLC plates (silica gel 60; Merck, Darmstadt, Germany), which were developed in $CHCl_3/CH_3OH$/triethylamine/H_2O (30:35:34:8, by vol.). The plates were dried at 180° C, stained by dipping in a 10% solution of $CuSO_4$ in 8% H_3PO_4, heated to 180° C within 7 min, and quantitated in a CD60 densitometer (Desaga, Heidelberg, Germany).

Pharmacokinetics. Blood samples from healthy female Wistar rats were drawn at 0.17, 0.5, 1, 3, 6, 24, 48, 115, and 170 h post administration. The maximal peak concentration (c_{max}) and peak time (t_{max}) were obtained from semilogarithmic concentration-time profiles (Fig. 1). The half-life ($t_{1/2}$) was calculated as ln2/k for oral application from the same profile. For i. v. injection, the distribution half-life (t_α) and the terminal elimination half-life (t_β) were obtained from the semilogarithmic concentration-time profiles by application of the residual method. The half-value duration (HVD) is the period during which the serum concentration is greater than 50% of the maximal concentration. The area under the serum concentration-time curve (AUC) was calculated using the trapezoidal rule and extrapolated to infinity. The terminal part of the AUC beyond the last measured data point (c_{last}) was estimated as the product of t_β and c_{last} divided by ln2 for i. v. data and as the product of $t_{1/2}$ and c_{last} divided by ln2 for oral data. Free HePC is nearly completely resorbed from the intestine [28]. The total body clearance (Cl_{tot}) was calculated as the delivered dose (in micromoles of HePC per kilogram of body weight) divided by the AUC. For oral administration, the relative distribution volume (V_d) was determined as the product of the dose and the half-life divided by the product of ln2 and the AUC. For i. v. injection, the relative distribution volume (V_d) was calculated as the dose divided by the peak concentration (c_{max}).

Table 1. Pharmacokinetic parameters of HePC in different formulations. A single dose of 20 mg/kg HePC (50 µmol/kg) was given to healthy female Wistar rats in the free form as an aqueous solution (p. o.) and as SUVs (p. o. versus i. v. bolus). The SUVs consisted of HePC/Chol/PPG3PG in the molar ratio of 4:5:1. Parameters were obtained by fitting serum concentration-time data to equations as described in Materials and methods. Data represent mean values to 3 animals. ($t_{1/2}$ half-life, t_α distribution half-life, t_β terminal half-life, c_{max} peak serum concentration, *HVD* half-value duration, $AUC_{0-24\ h}$ area under the curve for 24 h, *AUC* area under the curve extrapolated to infinity, Cl_{tot} total body clearance, V_d relative distribution volume)

	Free HePC p. o.	SUVs p. o.	SUVs i. v.
$t_{1/2}$ (h)	48	120	t_α 0.5, t_β 16
Kinetics	1st order	1st order	Biphasic
c_{max} (mg/l)	22	14	73
t_{max} (h)	24	24	0
HVD (h)	62	142	0.6
$AUC_{0-24\ h}$ (mg h/l^{-1})	304	254	258
AUC (mg h/l^{-1})	1947	2659	358
Cl_{tot} (ml/min)	0.2	0.1	1.0
V_d (l/kg)	0.7	1.3	0.3

Tumor induction. Autochthonous, hormone-dependent mammary carcinomas were induced by three i. v. injections of 50 mg/kg MNU into the tail vein of female S-D rats [3].

Evaluation of tumor development. Individual tumor volumes were measured using Vernier calipers. The rats were weighed and palpated twice weekly. Rats showing a total tumor volume of more than 0.8 cm^3 were randomly allocated to experimental groups. Therapy was started immediately. The tumor volume in treated and untreated control rats was recorded during the entire experimental period. Antitumor activity was assessed by recording the number of discrete tumors and the mean tumor volume per rat until the end of the therapy. The median tumor volume determined for each group was expressed as a percentage of the untreated control value (T/C, %).

Results

Table 1 gives a summary of the pharmacokinetic parameters for HePC in different formulations after single p. o. and i. v. administration of equimolar drug doses in healthy Wistar rats. The serum concentration-time profiles are shown in Fig. 1. The best results were obtained with a two-compartment model for i. v. data, whereas for oral data a one-compartment model was satisfactory. After i. v. injection of HePC-SUVs, the elimination from serum occurred biphasically, with the initial distribution half-life (t_α) being 0.5 h and the slower elimination half-life (t_β) being 16 h.

After p. o. administration, a change in the pharmacokinetic parameters was observed for free and liposomal HePC, with peak concentrations occurring at 24 h after application, an indication of a relatively slow process of absorption. Interestingly, the peak concentration measured in serum after oral administration of free HePC exceeded that found for liposomal HePC. The AUC values calculated for liposomally encapsulated HePC slightly exceeded those

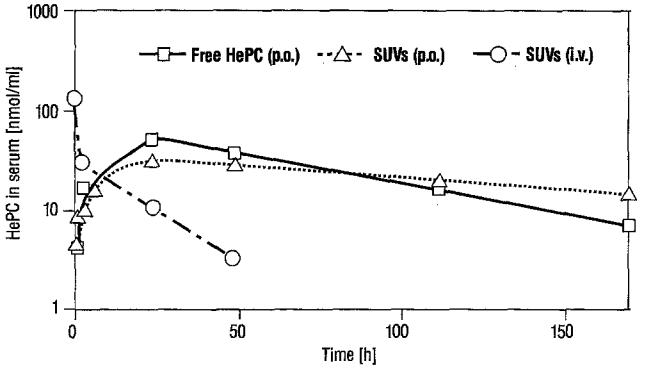

Fig. 1. Concentration-time profile of HePC in serum after the administration of a single dose of 20 mg/kg (50 µmol/kg) HePC to healthy Wistar rats. HePC was given in the free form (p. o.) and as SUVs (p. o., i. v.). The SUVs consisted of HePC/Chol/PPG3PG in the molar ratio of 4:5:1

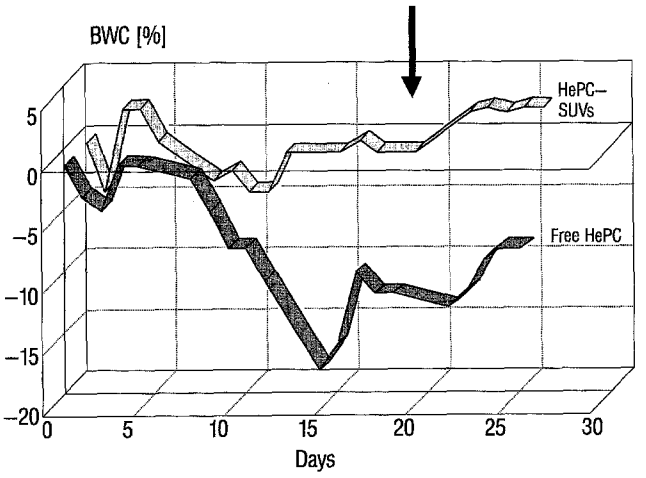

Fig. 2. BWC estimated as the difference in weight observed after the oral administration of HePC at a weekly dose of 50 µmol/kg to healthy Wistar rats and expressed as a percentage of the initial body weight. Data represent mean values (SD, ±10%) for 3 animals. HePC was given in the free form as an aqueous solution or as SUVs five times a week. The SUVs consisted of HePC/Chol/PPG3PG in the molar ratio of 4:5:1. Untreated control animals normally showed a weekly BWC of around +2% (*Arrow* change to pulverized feed)

determined for HePC given in the free form. Surprisingly, these AUC values were considerably higher than those resulting from i. v. injection of liposomal HePC. After oral use, the serum half-life of liposomal HePC (120 h) exceeded the half-life of free HePC (48 h). The V_d and Cl_{tot} values obtained for orally given free HePC and HePC-SUVs were low and showed no significant difference (Table 1).

Figure 2 shows the BWC observed in healthy Wistar rats following multiple oral dosing with free and liposomal HePC (weekly dose, 20 mg/kg; 4 weeks of treatment). Animals fed with free HePC showed an approximately 10% reduction in body weight, substantial gastrointestinal macroscopic damage, and slightly ruffled skin (data not shown). After 10 days, the food pellets were pulverized for all animals to facilitate ingestion. Over a period of 4 weeks following the oral administration of liposomal HePC, there was little change in body weight, no gastrointestinal lesion, and no ruffled skin. During the same period, untreated healthy control animals normally showed a weekly BWC of about +2%.

S-D rats bearing autochthonous, MNU-induced mammary carcinoma were used for evaluation of the antineoplastic efficacy of free and liposomal HePC. Therapy was stopped after 5 weeks, and the total observation period lasted for 10 weeks. A significant dose-response relationship in terms of tumor growth inhibition was obtained for orally given free HePC (Fig. 3). A weekly dose of about 100–150 mg/kg was found to be effective, and the T/C value was markedly reduced to 16% and 7% without therapy-related mortality. As compared with an oral dose of 150 mg/kg free HePC, the optimal i. v. dose of liposomal HePC (i. e. that producing significant tumor growth inhibition with no sign of toxicity or mortality) was only 80 mg/kg (Figs. 3, 4). A weekly i. v. dose of 160 mg/kg liposomal HePC led to an even more extensive tumor reduction, with complete remissions being observed in some animals (Fig. 3), but was

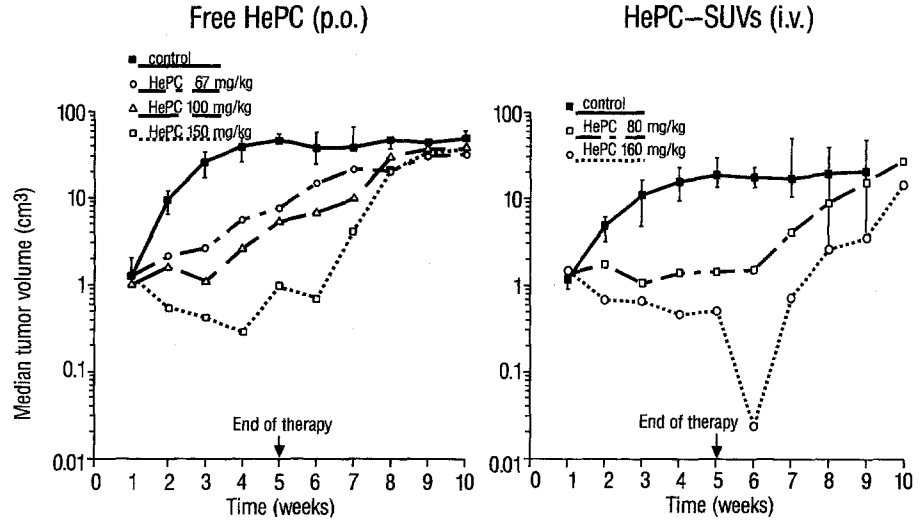

Fig. 3. Antineoplastic efficacy of HePC in rats bearing MNU-induced mammary carcinoma after 5 weeks of treatment. HePC was given in the free form (p. o.) and as SUVs (i. v.) at different weekly doses. The SUVs consisted of HePC/Chol/PPG3PG in the molar ratio of 4:5:1

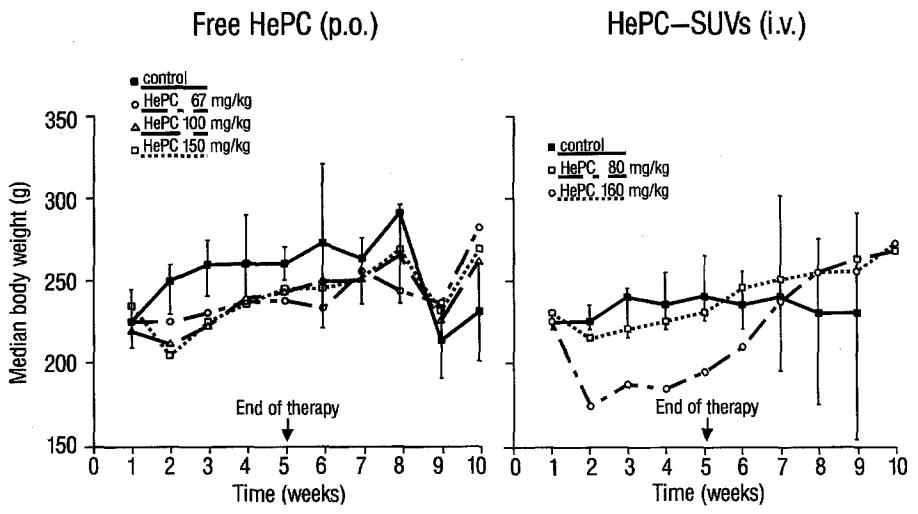

Fig. 4. Influence of HePC on the median body weight of rats bearing MNU-induced mammary carcinoma after 5 weeks of treatment. HePC was given in the free form (p. o.) and as SUVs (i. v.) at different weekly doses. The SUVs consisted of HePC/Chol/PPG3PG in the molar ratio of 4:5:1

poorly tolerated because it led to a decrease in body weight (Fig. 4) and increased the mortality to 20%. Cessation of therapy with free as well as liposomal HePC led to a regrowth of mammary carcinoma within 1 week (Fig. 3). The time course of the tumor-volume increase in treated rats was found to be similar to that in controls in both cases.

Discussion

Oral administration of liposomes has often been criticized because low pH values and digestive degradation can destroy the liposomal structure. A great deal of controversy exists concerning the oral use of liposomal drugs [1, 8–10, 12, 19, 24, 31, 34, 36, 38, 49]. Proper evaluation is difficult because of different absorption models, methods, and lipid compositions. We cannot determine from our experiments the fate of liposomes made from HePC, Chol, and PPG3PG. In the case of oral administration of HePC, the decreased loss of body weight and reduced gastrointestinal toxicity in healthy rats demonstrates that the liposomal drug was superior to the free drug. After oral administration of liposomal HePC, the concentration of non-lamellar-bound HePC seemed to be reduced in comparison with that of free HePC.

When given orally in the free form to healthy animals, HePC is almost completely resorbed from the intestine [28], most likely by a process of passive diffusion. Via chylomicrons and lymphatic capillaries, HePC might reach the blood mainly by way of the thoracic duct under bypass of the liver. In the blood, HePC is 90% bound to serum albumin [28] and shows a long half-life [46]. After oral application the bioavailability of liposomal HePC was even better than that of free HePC. In addition, we found a longer half-life and lower peak concentration for the liposomal drug. It seems that orally delivered liposomal HePC not only reduces the most common side effects but, because of the prolonged residence time at the site of absorption, also may serve as a depot. In contrast, i. v. liposomal HePC was eliminated rapidly from the circulation. We suspect that HePC-SUVs are not destroyed in the bloodstream but rather are eliminated as liposomes by macrophages in the liver. This behavior may account for the observation of lower $AUC_{i.v.}$ values relative to the AUC values determined after oral administration of free and liposomal HePC.

Independent of the mode of application or formulation, the relative distribution volumes for HePC are low, indicating distribution in the body water [30]. The pharmacokinetic values summarized in this publication for oral application differ from those described earlier [11, 46]. In this study we used a modified HPTLC method that produced a more sensitive and specific determination of alkylphosphocholines in tissues [27]. Additionally, a much smaller HePC dose was used, resulting in differing serum concentration-time profiles.

The effective oral dose of free HePC for antineoplastic efficacy has been given as 150 mg/kg per week [6], whereas the optimal i. v. dose for liposomal HePC is lower (80 mg/kg per week). Tumor regrowth after the end of the therapy probably results not from resistance but rather from newly arising tumors that develop continuously from the mammary chains after the original induction. Even after multiple i. v. injections of HePC-SUVs, neither thrombophlebitis, hemoglobinurea, nor hemolysis was observed. We propose that the liposomal formulation is a suitable i. v. delivery system for HePC. On the basis of these encouraging in vivo findings it would seem that oral administration of liposomes is superior to that of free HePC. We feel that these considerations may be important for other structurally related compounds and other areas of therapy such as treatment of protozoan diseases [29].

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