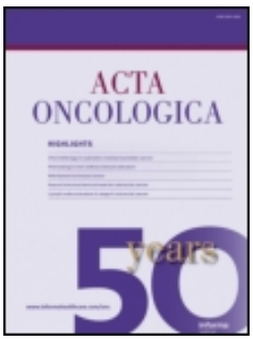

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FROM THE DEPARTMENT OF INTERNAL MEDICINE, DIVISION OF HEMATOLOGY/ONCOLOGY, UNIVERSITY OF GÖTTINGEN, MAX-PLANCK-INSTITUTE FOR BIOPHYSICAL CHEMISTRY, D-3400 GÖTTINGEN, AND ASTA PHARMA AG, D-4800 BIELEFELD, WEST GERMANY.

HEXADECYLPHOSPHOCHOLINE, A NEW ETHER LIPID ANALOGUE

Studies on the antineoplastic activity in vitro and in vivo

C. UNGER, W. DAMENZ, E. A. M. FLEER, D. J. KIM, A. BREISER, P. HILGARD, J. ENGEL, G. NAGEL and H. EIBL

Abstract

Hexadecylphosphocholine (He-PC) is a new compound synthesized according to the minimal structural requirements deducted from studies with other ether lipids. In vitro studies on He-PC revealed remarkable antineoplastic activity on HL60, U937, Raji and K562 leukemia cell lines. In addition, He-PC, applied orally, showed a superior effect in the treatment of dimethylbenzanthracene-induced rat mammary carcinomas when compared to intravenously administered cyclophosphamide. After oral application He-PC was well absorbed from the intestine and metabolized in the liver by phospholipases C and D. During a 5-week treatment no hematotoxic effects were detected. In a clinical pilot study on breast cancer patients with widespread skin involvement, topically applied He-PC showed skin tumor regressions without local or systemic side effects.

Key words: Hexadecylphosphocholine, ether lipids, antineoplastic activity.

Natural ester-lysolecithines (Fig. 1) are known to exist in small amounts in biological membranes. Twenty years ago, Westphal et al. at the Max-Planck-Institute in Freiburg found that very small amounts of exogenous lysolecithine strongly enhanced the phagocytic activity of peritoneal macrophages in vitro and in vivo (1).

Due to the very short half-life of these ester-lysolecithines Arnold et al. (2), Eibl et al. (3–5) and Weltzien & Westphal (6) synthesized lysolecithine ether analogues (Fig. 1) for studies of the macrophage stimulating processes. These compounds are very stable against most phospholipid metabolizing enzymes. Surprisingly, these alkyllysophospholipids showed strong direct cytolytic effects on various tumor cells in vitro and in vivo (7, 8).

Testing of the structure activity relationship among a variety of synthetic alkyllysophosphocholines showed that a long alkyl chain and a phosphocholine moiety may represent the minimal structural requirement for sufficient antineoplastic effects of the ether lipid analogues (9–11). This finding led to the synthesis of a new compound group, the alkylphosphocholines (Fig. 1) (12, 13).

In the following report, some preclinical and the first clinical investigations are presented with hexadecylphosphocholine (He-PC).

Material and Methods

Hexadecylphosphocholine was prepared according to the general procedures for the conversion of alcohols into the respective phosphocholines as described by Eibl & Woolley (14), and Eibl & Westphal (4). A Rf-value of 0.20 for the compound in the system chloroform/methanol/acetic acid/water 100:60:20:5 (by vol.) was observed. Hexadecylphospho[^{3}H-methyl]choline was obtained by methylation of the corresponding hexadecylphosphoethanolamine with [^{3}H]methyliodide (15). The resulting specific activity of hexadecylphospho[^{3}H-methyl]choline was 0.4 GBq/mmol/l.

Cell experiments. The in vitro cytostatic and cytotoxic activities of He-PC were determined in 4 human leukemia cell lines: HL60 and U937 (promyelocyte leukemia), Raji (Burkitt's lymphoma) and K562 (erythroleukemia). The cells were incubated for 72 h at 37°C and 5% CO_2 in RPMI medium with 10% fetal calf serum in different concentrations of He-PC for up to 72 h. The 50% growth inhibition dose (ID_{50}) after 48 h of incubation was determined by calculating the loss of [^{3}H]thymidine incorporation into

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cellular DNA. For the half lethal dose (LD_{50}) the trypan blue dye exclusion assay was used.

Animal experiments. The in vivo antineoplastic activity of He-PC was determined on dimethylbenzanthracene (DMBA)-induced rat mammary carcinomas (16) in comparison to cyclophosphamide as a standard cytotoxic agent. Daily oral treatments for 28 days with He-PC or with cyclophosphamide as a single i.v. dose respectively, were started when DMBA-induced mammary carcinomas of adult female Sprague Dawley rats attained a mass of 0.8–1.2 g.

To evaluate bone marrow toxicity, the white blood count of adult female Sprague Dawley rats was examined under oral treatment with He-PC administered in daily doses from 4.7 up to 46.4 mg/kg.

In organ distribution studies female NMRI mice (25 to 30 g weight) were used. For this purpose 0.07 MBq He-PC (specific activity: 1.85 MBq/μmol/l) was injected into the tail vein; oral applications with 0.19 MBq He-PC (specific activity: 0.37 MBq/μmol/l) were performed via a tube into the stomach. After the indicated time intervals the animals were killed, the organs removed, weighed, and the amount of radioactivity was determined as described earlier (17). To evaluate the uptake of He-PC in the various organs, the radioactivity was calculated as percentage of total dose per gram organ.

To investigate metabolism of He-PC in the liver, female NMRI mice were injected with 1.85 MBq/μmol/l He-PC (specific activity: 3.9 MBqμmol/l) into the tail vein. The animals were killed after 24 or 72 h respectively. The livers were removed and homogenized. Radioactive metabolites were determined by thin layer chromatography as described before (17, 18).

The first clinical studies on the topical treatment of skin metastases with hexadecylphosphocholine were performed. For better skin penetration He-PC was dissolved at a concentration of 20 mg/ml in a composition of water and alkylglycerols with different alkyl chain lengths (KASKADE, see Table 1). Patients with local recurrence of mammary carcinomas were included in the pilot study after their written consent. All patients had a history of exhaustive treatment including chemotherapy and local irradiation. Treatment was performed twice daily on the affected skin areas. In order to detect systemic side effects, blood and urine analyses, ECG and roentgenologic controls were performed.

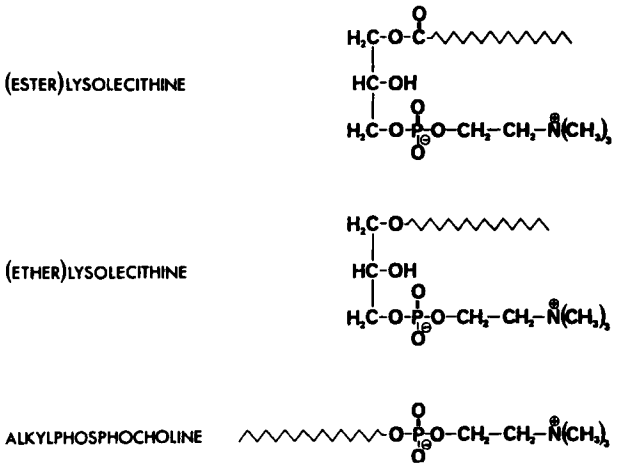

Fig. 1. Chemical structure of ester-lysolecithine, etherlysolecithine, and alkylphosphocholine.

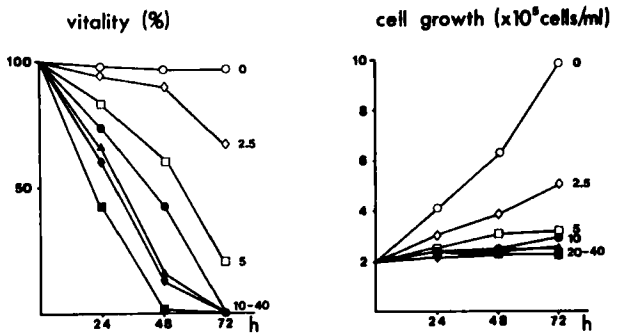

Fig. 2. He-PC dose dependant toxicity and inhibition of proliferation of the HL60 leukemia cell line. 2×10^5 cells were cultured for 24, 48 and 72 h in the presence of 2.5 to 40 μg/ml of He-PC; at each time point the number of total cells were counted for cell growth and the number of dead and alive cells were determined for cell vitality by trypan blue dye exclusion. Data represented the mean for 3 to 6 independant experiments; SD $<\pm10\%$.

Table 1

Composition of Kaskade. 20 mg hexadecylphosphocholine was dissolved in 1 ml of a formulation composed of water and the different alkylglycerols

Compound	% (w/v)
Nonylglycerol	17
Hexylglycerol	17
Propylglycerol	33
H_2O	33

Results and Discussion

Alkylphosphocholines, a new group of antineoplastic agents, are derived from cytotoxic alkyllysophosphocholines (12, 13). In vitro investigations of He-PC, a compound representative of alkylphosphocholines revealed concentration dependant cytotoxicity and growth inhibition shown in Fig. 2 for HL60 cells. ID_{50} and LD_{50} values were calculated from dose response curves after 48 h of incubation (Table 2) when cell growth was in a logarithmic phase. The data indicate that HL60 and U937 cells were of high sensitivity, Raji of intermediate and K562 of low sensitivity against He-PC. The ranking order of susceptibility of the 4 cell lines was similar in both systems and corresponds to results obtained for alkyllysophosphocho-

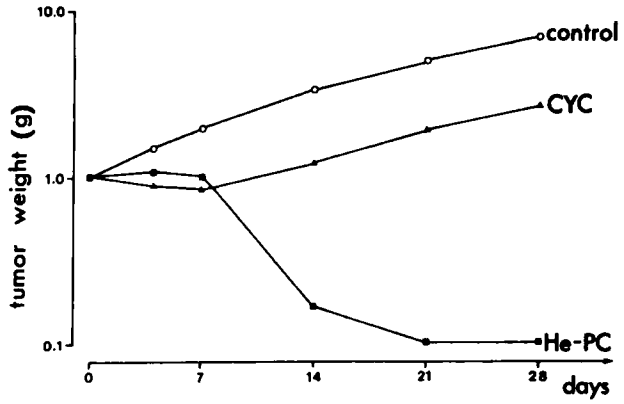

Fig. 3. Antineoplastic activity of He-PC in comparison to cyclophosphamide on DMBA-induced rat mammary carcinoma. Doses: He-PC 31.6 mg/kg body weight daily p.o. for 28 days; cyclophosphamide: 147 mg/kg as a single i.v. injection (maximally tolerated dosage).

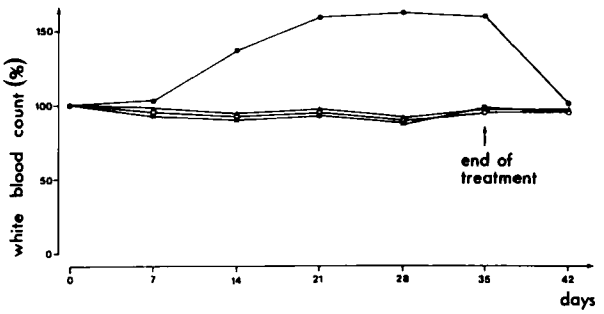

Fig. 4. Influence of He-PC treatment on white blood count of adult female Sprague-Dawley rats. He-PC was given orally at doses of 4.64 (▲—▲), 14.7 (■—■) and 46.4 (●—●) mg/kg per day for 35 days. Control (○—○).

Table 2

Differential sensitivity of HL60, U937, Raji and K562 leukemia cell lines to He-PC. ID_{50} values were obtained from [^3H]thymidine incorporation data after 48 h of incubation; the LD_{50} values were determined from the 48-h values as described in Fig. 2

Cell line	He-PC (μg/ml)	
	ID_{50}	LD_{50}
HL60	5.0	7.7
U937	3.0	16.0
Raji	10.0	26.0
K562	15.0	>40.0

lines, such as 1-0-octadecyl-2-0-methyl-rac-glycero-3-phosphocholine (OM-GPC) in earlier experiments (19).

The in vivo antitumoral effect of He-PC was studied in DMBA-induced rat mammary carcinomas in comparison to cyclophosphamide, which is known to be an active compound in this tumor model (20). He-PC was given orally at a dose of 31.6 mg/kg per day over 4 weeks, whereas cyclophosphamide was given as a single i.v. injection at the maximally tolerated dosage of 147 mg/kg. Fig. 3 demonstrates the superior effect of He-PC over cyclophosphamide. He-PC resulted in a complete disappearance of the tumor nodules during the treatment period. Within 3 weeks after cessation of the therapy the tumors started to regrow. Whether or not this represents regrowth of suppressed or development of new tumors is currently under investigation.

Table 3

Organ distribution of He-PC after oral administration in NMRI mice. Data represent percentage of total radioactivity/g organ (wet weight). SD<20%, 3–10 experiments for each time point. For details see 'Material and Methods'

Time	1 h	5 h	24 h	48 h	96 h	196 h
Blood	0.4	1.8	1.8	1.5	0.9	0.9
Stomach	7.5	4.4	5.0	3.6	2.6	1.9
Small int.	17.4	24.5	8.4	4.0	2.8	1.8
Large int.	0.4	3.3	3.8	2.7	2.0	1.3
Kidney	2.0	23.0	34.0	26.2	14.5	11.8
Lung	0.8	8.1	8.3	6.4	4.1	3.0
Liver	1.3	10.8	14.7	12.4	10.2	5.8

He-PC treatment was well tolerated and no overt toxic symptoms were noted; in particular no weight loss could be observed in the animals. Recently, high therapeutic activity of He-PC was also reported for the methylnitrosourea-induced rat mammary carcinomas, whereas 2 transplantable mammary carcinomas and authochthonous benzopyrene-induced sarcomas in the rat exhibited low-grade sensitivity to He-PC (21). It is noteworthy that in contrast to most cytostatics, He-PC treatment did not result in any suppression of the white blood count (Fig. 4). The leukocyte count, expressed as percentage of normal values, remained unchanged up to a dose of 14.7 mg/kg per day when given orally, whereas the highly effective dose of 46.4 mg/kg per day showed a surprising increase in leukocyte count during the treatment period. After finishing the treatment, the leukocyte count decreased to normal values within one week. Since bone marrow examination did not show augmentation of granulocyte precursor cells (22), it seems likely that an activation of the marginal leukocyte pool in the periphery may be responsible for this increase in leukocyte count.

Biodistribution studies after oral administration of He-PC in mice clearly demonstrated high absorption rates from the intestine (17), permitting oral administration in treatment regimens. This finding is in line with other reports showing that OM-GPC (23) and 1-hexadecylmercapto-2-methoxymethyl-rac-glycero-3-phosphocholine (24) are well absorbed from the intestinal tract. As is also known for OM-GPC (23), the highest accumulation of He-PC was found to occur in liver, kidney and lungs (Table 3).

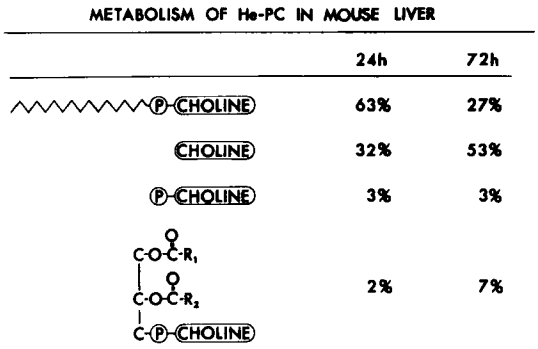

Fig. 5. Metabolism of hexadecylphospho[^{3}H-methyl]choline in mouse liver after i.v. administration. See 'Material and Methods' for details. Data represent the mean values of 2 experiment for the indicated times.

Metabolism of He-PC was investigated in the mouse liver. After oral administration of hexadecylphospho [^{3}H-methyl]choline, a time dependant formation of radiolabelled choline, phosphocholine and 1,2-diacylglycerophosphocholine was observed (Fig. 5), which suggests the importance of phospholipases C and D in He-PC catabolism. Studies on hydrolysis of He-PC in vitro, carried out with phospholipase C (bacillus cereus) and phospholipase D (partially purified from cabbage) support the finding that He-PC is a substrate for these enzymes (9, 11). Formation of 1,2-diacylglycerophosphocholine, labelled in the polar head group, is consistent with earlier results obtained with OM-GPC (15, 18). In the latter study it was shown that the labelled phosphocholine group was transferred from OM-GPC into 1,2-diacylglycerol, which resulted in the formation of phosphatidylcholine.

The favorable results obtained on tumor response in chemically induced autochthonous rat mammary carcinomas prompted us to investigate the antitumoral effect of He-PC in the topical treatment of skin metastases in humans. For this purpose He-PC was dissolved in short chain alkylglycerols (Table 1), since it was recently demonstrated that these compounds are able to increase drug penetration through biological membranes (25, 26). Hence, it is possible that these compounds also improve penetration of He-PC into the deeper compartments of the skin. In addition, hexyl- and nonylglycerol display considerable growth inhibition properties on leukemia cells in vitro (to be published).

In the clinical pilot study 14 patients with local recurrence of breast carcinoma were included and treated twice daily by topical application. The treatment was well tolerated and apart from minor side effects, such as reddening, flaking and itching, no systemic toxicity was observed. Two patients showed a complete remission lasting 4 and 6 months, and one patient showed a partial remission for 7 months. Skin biopsies taken from invaded and normal skin after treatment revealed a moderate increase in the formation of collagen fibers without affecting the normal skin architecture.

Therefore, topically applied ether lipids may represent a safe and effective treatment of skin metastases. Based on these promising observations, regular phase I trial studies (oral and topical applications) have been started.

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