Eur J Cancer Clin Oncol, Vol. 24, No. 9, pp. 1457–1461, 1988.
Printed in Great Britain

0277-5379/88$3.00 + 0.00

Characterization of the Antitumor Activity of Hexadecylphosphocholine (D 18506)

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Abstract—*Hexadecylphosphocholine (HPC) differs from ether lipids with known antitumor activity by its lack of the glycerol part. In the experiments described here HPC revealed outstanding antitumor activity in dimethylbenzanthracene (DMBA)-induced rat mammary tumors. A dose–response relationship was seen after daily oral treatment with complete suppression of tumor growth at doses of 46.4 mg/kg/day. There was no schedule dependence and the therapeutic efficacy was independent of the tumor weight at the initiation of therapy. Another autochthonous tumor, the benzo[a]pyrene-induced sarcoma of the rat did not respond to HPC treatment, indicating a highly selective spectrum of activity of the test compound. In comparison to an optimal single dose of cyclophosphamide, a single high dose of HPC was considerably more active against the DMBA tumor. At therapeutic dose levels no major toxicity of HPC was observed. Bone marrow suppression was not encountered, on the contrary, at high doses leukocytosis became apparent. The available pharmacological and toxicological data suggest that HPC may be useful in the treatment of human cancer.*

INTRODUCTION

ETHER LIPIDS have several biological functions as important components of mammalian cells and considerable interest has recently focussed on the 'platelet activating factor' (PAF; 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine), which appears to be of pathogenic significance in the mediation of inflammatory and allergic reactions. Several analogs of PAF, which do not have platelet aggregating activity, are potent cytotoxic agents for a variety of cell types [1]. Lysophosphatidyl-choline (LPC) is an important intermediate in lipid metabolism and since a role was postulated for LPC in the macrophage mediated immune response, alkyl analogs of LPC with immunomodulatory properties were synthesized [2]. In addition, these compounds were, similarly to the PAF analogs, cytotoxic. Racemic 1-octadecyl-2-methyl-glycero-3-phosphocholine (Et-18-OCH_3) was the first of the LPC analogs to undergo extensive preclinical evaluation. It served as the prototype for a new class of anticancer agents, with activity in a variety of experimental tumor models [3]. The precise mode of the tumor inhibitory action of alkyllysophospholipids is not yet fully understood. The biological activities at the level of the malignant target cell may include direct cytotoxicity [4], induction of differentiation [5], inhibition of invasion [6] and activation of cytotoxic macrophages [7]. Recently alkylphosphocholines were investigated for their antitumor activity and hexadecylphosphocholine (HPC, D 18506, Fig. 1) was found to have remarkable antineoplastic activity *in vitro* and *in vivo* [8, 9]. The present communication describes some of the pharmacological and toxicological data of HPC obtained in our laboratories and it is hoped that they might be a useful contribution for the development of trial strategies in the clinical evaluation of this class of antitumor agents.

Accepted 28 April 1988.
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MATERIALS AND METHODS

1. *Animals and tumor induction*

Virgin female Sprague–Dawley rats (Moellegaards Breeding Center, Ejby, Denmark) were used throughout the experiments. The animals were kept under specific pathogen-free (SPF) conditions, were fed with standard pellet diet (Altromin® 1324) and had unrestricted water supply (acidified to pH 3). Multiple mammary carcinomas were induced by 20 mg dimethylbenzanthracene (Serva, Heidelberg), dissolved in 1 ml of olive oil and administered by gavage as a single dose to 50-day-old rats.

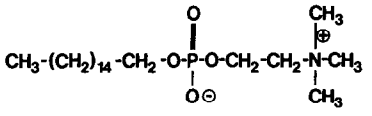

Fig. 1. Chemical structure of hexadecylphosphocholine (molecular weight 407.6).

Thirty to sixty-five days after induction the first tumors appeared along the lactic line. Benzo[*a*]-pyrene-sarcomas were induced by the subcutaneous injection of 5 mg benzo[*a*]pyrene (Serva, Heidelberg), dissolved in 1 ml olive oil, into the back of female rats (120–150 g). Rapidly growing solid tumors appeared at the injection site around day 95 after induction.

Tumor measurements were performed in weekly intervals according to the method described by Druckrey *et al.* [10]. In short, the tumor weight was estimated by palpation and by comparison of its volume with that of prefabricated plasticine models. The weight of the models was determined and converted into tumor weight by a factor which reflected the differences in the specific weight of tumor tissue and plasticine. In a large series of comparative measurements the standard deviation of this method was 5%.

2. *Treatments*

HPC, manufactured by Asta Pharma AG, with a chemical purity of greater than 98% was dissolved and diluted with distilled water. All control animals received 0.9% saline. Each animal was treated by administration of 2.15 ml solution/kg body wt through a stomach tube. Commercial grade cyclophosphamide (Asta Pharma AG) was dissolved in saline. In each of the experimental groups the body weight of the animals was determined before and twice weekly during treatment. In order to assess the true body weight the tumor weight was subtracted from the measured value. All deaths and clinical symptoms during treatment and the following 2 weeks period were recorded.

(a) Dose finding studies for the daily treatment with HPC were carried out in normal rats.
(b) Hemoglobin red cell count, platelet count, leukocyte count and blood smears were performed in a separate treatment experiment using standard techniques. Blood was taken from the sublingual vein of the rat.
(c) Studies of the dose–response relationship to HPC and its schedule dependence were begun when the tumor weight in an individual animal had reached 0.8–1.5 g. After discontinuation of the treatment the additional observation period was 2 weeks.
(d) To assess the efficacy of HPC therapy in small vs. large tumors, DMBA tumor-bearing animals were individually randomized into two treatment and two corresponding control groups. Treatment was started either at a tumor weight between 0.8 and 1.2 g or between 5.0 and 8.0 g.
(e) To compare the therapeutic efficacy of HPC with that of a standard drug, maximally tolerated single doses of HPC (215 mg/kg orally) and cyclophosphamide (147 mg/kg i.v.) were administered by the route which was previously established to give the optimal therapeutic results.
(f) When a tumor nodule became palpable at the site of benzo[*a*]pyrene injection, it grew extremely rapidly (average weight after 3 weeks 79 ± 12 g), and therefore the range of tumor weights for inclusion into a therapy experiment had to be extended to 1–6 g.

3. *Statistical analyses*

All test results were given as the median of individual data or as arithmetic means as indicated. The significance of the pairwise comparisons of treatment in each experiment versus control groups was established by the Mann–Whitney rank-sum test. Differences between tumor growth curves were analyzed by the distribution-free, multivariate rank test described by Koziol *et al.* [11].

RESULTS

In a five week daily oral treatment with HPC (six rats 230–270 g body wt per dose group) the maximally tolerated dose was 46.4 mg/kg/day, whereas 68.1 mg/kg resulted in several deaths (two out of six animals) already after 10 days of treatment. A frequent finding in animals on higher doses (46.4–68.1 mg/kg) was hair loss, in particular on the head. A transient reduction in body weight during the first week of treatment was observed at doses from 10 to 46.4 mg/kg ($P < 0.01$). At 68.1 mg/kg all animals experienced progressive weight loss under therapy.

No hematological toxicity was encountered after various doses of HPC during a 5 week treatment and an additional 1 week observation period. Hemoglobin, hematocrit and platelet count remained unchanged. In the highest dosage group (46.4 mg/kg) a significant increase in the total white blood cell count occurred during the treatment (Fig. 2). This was mainly due to an increase in the absolute numbers of mature granulocytes; the WBC count turned back to normal within a week after cessation of therapy.

The dose–response relationship of DMBA-induced tumors to HPC administered over 5 weeks is demonstrated in Fig. 3. By comparison of the tumor growth curves at the various dose levels it can be seen that the therapeutic effect is clearly related to the daily dose administered. Already

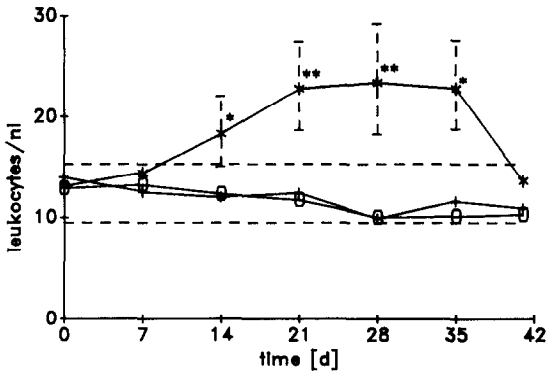

*Fig. 2. Leukocyte count (arithmetic means) in rats treated daily with HPC for 5 weeks. Test groups consisted of five animals. The normal range (arithmetic means ± S.D.) is indicated by the dashed lines (*P < 0.05; **P < 0.01). —+— 4.64 mg/kg/day; —o— 14.7 mg/kg/day; —*— 46.4 mg/kg/day (± S.D.).*

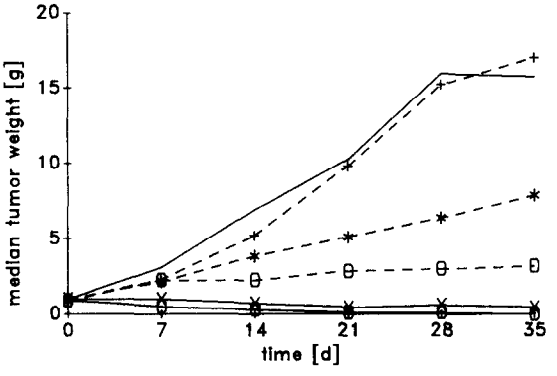

Fig. 3. Growth curves of DMBA-induced mammary tumors in rats under daily treatment with HPC in 5 weeks. Test groups consisted of 10 animals. —— control; —+— 2.15 mg/kg/day; —— 4.64 mg/kg/day; —o— 10 mg/kg/day; —×— 21.5 mg/kg/day; —o— 46.4 mg/kg/day.*

4.64 mg/kg resulted in a marked inhibition ($P < 0.01$) and complete growth retardation was seen at doses between 10 and 21.5 mg, whereas 46.4 mg consistently induced the regression of all tumors to below the level of palpability. The average numbers of tumors within the treatment groups are summarized in Table 1. As can be seen the reduction in numbers parallels the growth inhibition and reaches statistical significance at a dose level of 10 mg/kg ($P < 0.01$). Within 2 weeks after cessation of the therapy all tumors, including those in complete remission, started to regrow.

A single high dose of 200 mg/kg HPC given in weekly intervals proved to be as effective in suppressing tumor growth as five repeated daily low doses of 40 mg/kg or intermediate doses of 2 × 100 mg/kg per week. The tumor growth curves of the various groups are shown in Fig. 4. There was no significant difference between the tumor growth curves of any of the three treatment schedules.

The influence of the tumor size on the therapeutic effect of HPC is shown in Table 2. As can be seen,

Table 1. Effect of HPC (daily treatment p.o., 5 weeks) on the number of tumor nodules formed in DMBA-treated rats

Dose(mg/kg)	Median number of tumor nodules
0	10.5
2.15	15.0
4.64	13.5
10.0	5.0**
21.5	2.5**
46.4	0.0**

**$P < 0.01$.

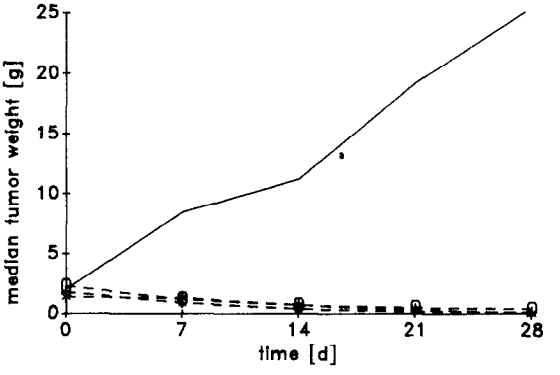

Fig. 4. Growth curves of DMBA-induced mammary tumors treated for 4 weeks with different dose schedules for HPC (200 mg/kg/week). Test groups consisted of 10 animals. —— control; —o— 5 weekly doses; —— 2 weekly doses; —+— 1 weekly dose.*

Table 2. Effect of a single dose of HPC (215 mg/kg) on tumor weight in DMBA-treated rats. The treatment started when the tumor weight was in the range of 0.8–1.2 g (small tumors) or greater than 5 g (large tumors)

	Dose (mg/kg)	Median tumor weight (g) day 0	day 7	day 14
Small tumors	0	1.0	3.0	6.9
	215	0.8	0.4**	0.5**
Large tumors	0	6.9	11.2	17.4
	215	6.2	3.1**	3.5**

**$P < 0.01$ vs. control.

the response for a given dose of HPC was similar in large and small tumors.

Figure 5 shows the DMBA tumor growth curves of animals treated with optimal therapeutic single doses of HPC and cyclophosphamide. It is obvious that HPC induces a significantly better tumor response in this particular experimental model.

Benzo[*a*]pyrene induced tumors were only marginally influenced by HPC. The growth curves during the daily treatment with 46.4 mg/kg were almost identical to those of the controls. No statistically significant effect of the treatment could be established.

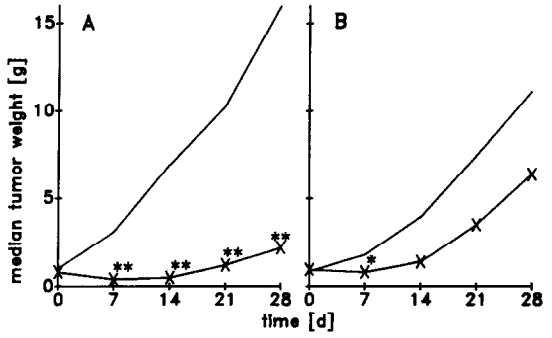

Fig. 5. Growth curves of DMBA-induced mammary tumors treated with (A) HPC (215 mg/kg *p.o.*) (×—×) *and controls* (—); *(B) cyclophosphamide* (147 mg/kg *i.v.*) (×—×) *and controls* (—). *Test groups consisted of* 10 *animals.* (*$P < 0.05$; **$P < 0.01$).

DISCUSSION

The cytotoxic properties of PAF and other alkyl-lysophospholipids were originally explained by interference of these compounds with the renewal of the tumor cell membrane phospholipid bilayer [12]. This hypothesis was supplemented by the work of Soodsma *et al.* [13] who demonstrated that transplantable tumors were deficient of *O*-alkyl-cleavage enzymes suggesting that they were unable to degrade toxic ether lipids. Since normal liver cells were rich in these enzymes, the finding indicated a certain selectivity of the cytotoxic action of ether lipids for tumor cells.

Studies with Et-18-OCH_3 (1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine) later revealed that, although this compound was a potent and selective cytotoxic agent, it was not a substrate for alkylglyceromonooxygenase, the major *O*-alkyl cleavage enzyme [14]. Subsequent investigations identified phosphatidylcholine as a major metabolite of Et-18-OCH_3, indicating a role of phospholipase C in the *in vivo* degradation of alkyllysophospholipids. Since the amount of phosphatidylcholine formed was strictly correlated to the *in vitro* cytotoxicity of the compound, the authors started to look for simpler molecular structures which would also be a substrate for phospholipase C [8]. The alkylphosphocholines fulfilled these requirements and HPC was soon discovered to be one of the most potent antitumor compounds in this series, having strong antineoplastic activity in MNU-induced mammary carcinoma of the rat [9].

The above findings motivated us to further investigate the antineoplastic activity of HPC in another carcinogen-induced mammary tumor of the rat. In DMBA rat mammary tumors a classical dose–response relationship was seen with complete responses constantly observed at the highest dose levels. Interestingly, all animals in which a complete disappearance of macroscopic tumors had occurred, relapsed within a short time after cessation of therapy. Preliminary pharmacokinetic data using a compound in which the choline was ^{3}H-labelled, demonstrated, as expected, that the substance was metabolized and that its half-life or that of one of its metabolites was rather long [15]. This would be consistent with the apparent lack of schedule dependence demonstrated in our experiments. If substantiated in humans, this observation may be of considerable clinical importance since it suggests that a high flexibility can be applied in finding the therapeutically optimal and best tolerated dose schedule.

In all therapeutic experiments described here HPC was extremely well tolerated. However, the 5 week tolerance study in rats indicated that there was a critical dose level at which major toxicities rapidly appeared. Since the therapeutic efficacy on the other hand is evident over a wide dose range, it was never necessary to treat at maximally tolerated doses in order to achieve significant antitumor activity. Compared to conventional chemotherapy, HPC appears to have a reasonably broad therapeutic range. In the autochthonous DMBA tumor model cyclophosphamide at its optimal dose level was rather ineffective if compared to HPC, and this finding might indeed suggest a spectrum of activity different from conventional cytostatics. In addition, no evidence for hematological toxicity was found in our studies. An intriguing and hitherto unexplained finding was the increase in the granulocyte count during treatment with the highest dose. The lack of bone marrow suppression in therapeutic doses makes HPC an interesting candidate for combination with cytotoxic chemotherapy.

Finally it must be stressed that the activity of HPC against experimental tumors appeared to be extremely selective. The transplantable rat tumors such as the DS-carcinosarcoma, AH 13s sarcoma, and the L5222 leukemia, as well as mouse tumors (L1210, P388, Lewis lung, B16 melanoma) maintained in our laboratories were not sensitive to any of the dose schedules described here [16]. The autochthonous benzo[*a*]pyrene-induced sarcoma of the rat was equally insensitive to HPC and therefore one can probably exclude a unique responsiveness of immunogenic carcinogen-induced tumors. Rillema's recent finding of high levels of phospholipase C activity in DMBA-induced rat mammary tumor tissue [17] is consistent with the above-mentioned hypothesis for a role of phospholipase C in the generation of cytotoxic metabolites from hexadecylphosphocholine. The fact that the classical tumor systems normally employed in drug development for the characterization of cytotoxic compounds do not reveal the antitumor activity of HPC, makes it an interesting candidate for further experimental and clinical evaluation. It remains to be seen, however, whether or not carcinogen-induced mammary cancers of the rat are predictive models for human malignant disease.

Acknowledgements—The skillful technical assistance of Mrs. M. Elliger, Mrs. K. Fenneberg, Mrs. B. Piening and Mr. H. Holtei is gratefully acknowledged. The analyses of the test compound were carried out by Dr. Wolf-Heuss, Department of Analysis, and Dr. R. Junge, Department of Biometrics, both Asta Pharma AG, provided assistance in the statistical evaluation of the test results. The present work was generously supported by the Federal Ministry of Research and Technology (BMFT), Bonn-Bad Godesberg, F.R.G.

REFERENCES

1. Hoffman DR, Hajdu J, Snyder F. Cytotoxicity of platelet activating factor and related alkyl-phospholipid analogs in human leukemia cells, polymorphonuclear neutrophils, and skin fibroblasts. *Blood* 1984, **63**, 545–552.
2. Munder PG, Fischer H, Weltzien HU, Oettgen HF, Westphal O. Lysolecithin analogs: a new class of immunopotentiators with antitumor activity. *Proc Am Ass Cancer Res* 1976, **17**, 174.
3. Berdel WE, Bausert WRE, Fink U, Rastetter J, Munder PG. Anti-tumor action of alkyl-lysophospholipids (review). *Anticancer Res* 1981, **1**, 345–352.
4. Berdel WE, Fink U, Egger B, Reichert A, Munder PG, Rastetter J. Inhibition by alkyl-lysophospholipids of tritiated thymidine uptake in cells of human malignant urologic tumors. *J Natl Cancer Inst* 1981, **66**, 813–817.
5. Honma Y, Kasukabe T, Hozumi M, Tsushima S, Nomura H. Induction of differentiation of cultured human and mouse myeloid leukemia cells by alkyllysophospholipids. *Cancer Res* 1981, **41**, 3211–3216.
6. Storme G, Berdel WE, Bruyneel R, De Bruyne G, Mareel M. Antiinvasive effect of an alkyl-lysophospholipid ET-18-OCH_3 (racemic 1-octa-decyl-2-methyl-glycero-3-phospho-choline) which has no microtubule inhibitor activity. *Proc Am Ass Cancer Res* 1983, **24**, 31.
7. Berdel WE, Bausert WR, Weltzien HU, Modolell ML, Widmann KH, Munder PG. The influence of alkyl-lysophospholipids and lysophospholipid-activated macrophages on the development of metastases of 3-Lewis lung carcinoma. *Eur J Cancer* 1980, **16**, 1199–1204.
8. Fleer EAM, Unger C, Kim D-J, Eibl H. Metabolism of ether phospholipids and analogs in neoplastic cells. *Lipids* 1987, **22**, 856–861.
9. Scherf HR, Schuler B, Berger MR, Schmähl D. Therapeutic activity of ET-18OCH_3 and hexadecylphosphocholine against mammary tumors in BD-VI rats. *Lipids* 1987, **22**, 927–929.
10. Druckrey H, Steinhoff D, Nakayama M, Preussmann R, Anger K. Experimentelle Beiträge zum Dosis-Problem in der Krebs-Chemotherapie und zur Wirkungsweise von Endoxan. *Dtsch med Wschr* 1963, **88**, 10–14.
11. Koziol JA, Maxwell DA, Fukushima M, Colmerauer MEM, Pilch YH. A distribution-free test for tumor-growth curve analyses with application to an animal tumor immunotherapy experiment. *Biometrics* 1981, **37**, 383–390.
12. Modolell M, Andreesen R, Pahlke W, Brugger U, Munder PG. Disturbance of phospholipid metabolism during the selective destruction of tumor cells induced by alkyl-lysophospho-lipids. *Cancer Res* 1979, **39**, 4681–4686.
13. Soodsma JF, Piantadosi C, Snyder F. The biocleavage of alkyl glyceryl ethers in Morris hepatomas and other transplantable neoplasms. *Cancer Res* 1970, **30**, 309–311.
14. Unger C, Eibl H, von Heyden H-W, Nagel GA. New assay for the *O*-alkyl cleavage enzyme with alkyl-lysophospholipids as substrates. *Cancer Res* 1985, **45**, 616–618.
15. Breiser A, Kim D-J, Fleer EAM *et al.* Distribution and metabolism of hexadecyl-phosphocholine in mice. *Lipids* 1987, **22**, 925–926.
16. Hilgard P. Unpublished observations.
17. Rillema JA. Phospholipase C activity in normal rat mammary tissues and in DMBA-induced rat mammary tumors. *Proc Soc Exp Biol Med* 1986, **181**, 450–453.