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Antitumor effects of alkylphosphocholines in different murine tumor models: use of liposomal preparations

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Hexadecylphosphocholine (HPC) and its analogs with a longer alkyl chain (C_{18} and C_{20}) were examined for antineoplastic activity in the murine tumor models P388 leukemia, B 16 melanoma, the mammary carcinoma C3H and Ca 755, and the human MT-1 mammary tumor in nude mice. The maximum tolerated doses were determined and found to be higher in mice than in rats. The toxicity of the alkylphosphocholines increases with chain length. The murine mammary carcinoma C3H and the human MT-1 mammary carcinoma showed response to HPC whereas the classical screening models did not respond to the synthetic lipids. Furthermore, HPC showed activity in a mitoxantrone-resistant P388 leukemia. Treated/control values between 120 and 160% in survival time could be obtained following a daily application of the lipid. Examination of the activity of possible cleavage products of HPC gave no information about the mechanism of action of the used etherlipids. Liposomes with encapsulated mitoxantrone, formed from alkylphosphocholines, cholesterol and dicetylphosphate had the same activity against P388 mouse leukemia as the free drug. The hemolytic activity of the three lipids tested *in vivo* was assumed to be related to toxic deaths of single animals; hemolytic activity was observed to be sometimes independent of schedule and dose.

Key words: Alkylphospholipid, eicosanylphosphocholine, etherlipid, hexadecylphosphocholine, liposomes, mammary carcinoma, mitoxantrone, octadecylphospholine, P388 leukemia, resistance.

Introduction

Systematical investigations of the structure–activity relation of the classical alkyl-lysophospholipids led to the development of a new generation of etherlipids, which showed the same physiological activity as the well-known Et-18-OCH_3 or Ilmofosin, but have a very simple structure.[1,2] First results were demonstrated with hexadecylphosphocholine (HPC, Figure 1).

This compound was tested extensively for the characterization of its toxicity, dose response, pharmacokinetics, metabolism and antitumor activity.[1–5] The best therapeutic results were obtained in chemically induced mammary tumors of the rat by oral treatment with HPC using a dose of 46.4 mg/kg/day.[3] Therapeutic results in other classical tumor models *in vivo* such as the P388 leukemia and the B 16 melanoma were disappointing.[4] Because of the good results in mammary tumors, HPC has now been evaluated in clinical trials for the first time.[6] It was the aim of our work to examine the activity of HPC and its analogs with longer alkyl chains in various murine tumor models *in vivo*.

Additionally, these substances were used as constituents of liposomal preparations with an encapsulated cytostatic.

Materials and methods

Substances

The alkylphosphocholines HPC, octadecylphosphocholine (OPC) and eicosanylphosphocholine (EPC) were synthesized as described elsewhere (Jungmann, S *et al.*, manuscript in preparation) and showed chromatographically a single spot (R_f 0.15, developing system chloroform/methanol/aqueous ammonia 78/30/4.7 v/v).

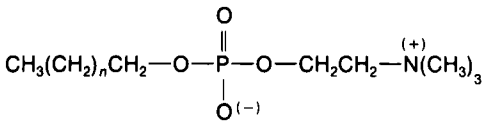

Figure 1. Structure of etherlipids used: hexadecylphosphocholine (HPC), $n = 14$; octadecylphosphocholine (OPC), $n = 16$ and eicosanylphosphocholine (EPC), $n = 18$.

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Table 1. Tumor models and mice

Tumor model	Mice	Inoculation[a]	Evaluation parameter
P388 leukemia	(C57B1/6 × DBA/2)F1 = BDF1, female	i.p.	MDST[b]
B 16 melanoma	(C57B1/6 × DBA/2)F1 = BDF1, female	i.m.	MDST, tumor growth
C3H mammary carcinoma	(C3H × Balb/c)F1 female	i.m.	Tumor growth
Ca 755 mammary carcinoma	(C57B1/6 × DBA/2)F1 = BDF1, female	i.m.	MDST, tumor growth
P388/Mitox	(C57B1/6 × DBA/2)F1	i.p.	MDST
MT-1	Bln: NMRI nu/nu	s.c.	Tumor growth

[a] i.p. = intraperitoneal; i.m. = intramuscular; s.c. = subcutaneous.
[b] Median survival time in days.

Liposomes were prepared according to Szoka.[7] The required amount of alkylphosphocholine, cholesterol (Merck, Darmstadt, Germany) and dicetylphosphate (Serva, Heidelberg, Germany) were mixed in a ratio of 1:1:0.25 and dissolved in chloroform, dried in a round-bottom flask under rotary evaporation for 2 h *in vacuo*. Multilamellar vesicles were formed by resuspending the lipid film with the solution of the pharmacon (mitoxantrone) in PBS and gentle shaking overnight.

Mice and tumor models

We used the mice and tumor models described in Table 1. The syngeneic murine tumors P388 leukemia, B 16 melanoma, and Ca 755 mammary carcinoma were transplanted in an internationally accustomed manner.[8] The C3H mammary carcinoma was used, taking the first transplant generation from spontaneously developing tumors in female C3H mice. Treatment in this model, in the Ca 755 and the human MT-1 mammary carcinoma was started when palpable tumors were noticed.

The P388/Mitox is a mitoxantrone-resistant P388 leukemia, showing multidrug resistance.[9] Mice were purchased in SPF quality (Biomodelle GmbH, Schönwalde) and held under low-germ conventional conditions (22°C room temperature, 60% relative humidity). They received autoclaved standard diet and tap water *ad libitum*. The corresponding evaluation parameters of treated groups were compared to the control group and expressed as treated/control (T/C) values in percent.

Each group consisted of 6–10 mice. Treatment schedules and routes of substance administration are documented in the tables. All substances were freshly dissolved in sterile physiologic saline with 10% Tween 80. The same solvent was also used for the treatment of control groups. Administration volume was 0.2 mg/20 g body weight. Animals were inspected twice daily. Individual survival times were registered and median survival times (MDST) in days calculated per group. All animals dying before controls were registered as toxic deaths. Tumor diameters were measured twice weekly with a caliper-like mechanical instrument and tumor volume V' was calculated according to the formula

$$V' = \frac{\text{length} \times \text{width}^2}{2}$$

Body weight change (BWC) as toxic parameter was registered between the first day of treatment and 3–4 days later and expressed as a percentage. White blood cells (WBC) were counted in peripheral blood 3 days after treatment.

Hemolysis

For determination of hemolytic capacity of ether lipids 30 minutes after i.v. injection of the substance, blood was collected and centrifuged. The concentration of hemoglobin in serum was measured according to the German Pharmacopeia. The extinction at 540 nm is used as quantitative measure.

Results

The maximum tolerated doses (MTD) of the three alkylphosphocholines following different treatment schedules in tumor-bearing mice can be seen in Table 2. In regard to HPC, there was no clear difference between the results from single or repeated, enteral or parenteral application. The MTD was always about 100 mg/kg/day. Even when lower doses were used, single deaths were observed, and no clear dose-mortality relation was registered. Lethally treated animals died within 1–3 days both after i.p. and oral application and showed signs of

Table 2. Maximum tolerated dose (MTD) of hexadecylphosphocholine (HPC), octadecylphosphocholine (OPC) and eicosanylphosphocholine (EPC) in female tumor-bearing BDF1 mice

Substance	Schedule (day)	Route[a]	MTD	
			(mg/kg/d)	(μmol/kg/d)
HPC	1	i.p.	100–200	245–491
	1–4	i.p.	100	245
	1–4	or.	100	245
OPC	1	i.p.	100–200	229–459
	1–4	i.p.	50	115
	1–4, 7–11	i.p.	20–25	49–57
EPC	1	i.p.	100–200	216–431
	1–4	i.p.	25	54
	1–4, 7–11	i.p.	20–25	43–54

[a] i.p. = intraperitoneal; or. = oral.

inflammation in the gut. The same also held true for OPC and EPC with the exception that multiple doses were clearly tolerated worse than a single one. A prolongation of the alkyl chain probably leads to higher and/or longer-lasting toxicity.

To estimate the antineoplastic efficacy of HPC we used four different murine and one human tumor model (Table 3). Three were mammary carcinomas, as experimental and clinical experience showed significant activity in this tumor localization.[3,4,6] In our studies HPC was active in the murine C3H and the human MT-1 mammary carcinoma, but not in the Ca 755 model. In the C3H model, HPC showed significant tumor volume inhibitions both following intratumoral or oral treatment, with a higher absolute inhibition after enteral use. In addition, no clear-cut relation could be demonstrated in these

Table 3. Antitumor efficacy of HPC in different murine tumor models

Tumor model	Dose (mg/kg/d)	Schedule (day)	Route[a]	Toxic/total	Survival T/C[b] (%)	Tumor growth[c] T/C(%)	BWC[d] (%)
P388 i.p.	200	1	i.p.	4/4	40		−6
	100	1	i.p.	0/10	100		−1
	10	1	i.p.	0/6	100		+5
	100	1–4	i.p.	0/10	100		−8
	50	1–4	i.p.	0/6	100		−8
	25	1–4	i.p.	0/6	100		−8
C3H Mamma i.m.	100	48–59	i.t.	7/10		90+	−5
	75	47–59	i.t.	0/10		114	+4
	50	48–59	i.t.	0/10		78+	+8
	25	33–44	i.t.	0/10		67	+1
	100	33–44	or.	6/10		32+	−6
	75	47–59	or.	0/10		36+	+9
	50	48–59	or.	1/10		77+	+5
	25	33–44	or.	2/10		47	0
Ca 755 i.m.	100	10–22	i.t.	0/10	100	127	1
	75	10–22	i.t.	0/10	125	104	5
	100	10–22	or.	0/10	78	110	0
	75	10–22	or.	0/10	102	91	2
P388/Mitox i.p.	100	1–4	i.p.	3/8	120		−3
	50	1–4	i.p.	0/8	135+		1
	100	1–4	or.	0/7	160+		−10
	50	1–4	or.	0/8	125+		3
	125	1–4	or.	2/9	140+		−11
	100	1–4	or.	0/9	140+		−6
	75	1–4	or.	0/10	140		0
MT-1 Mamma s.c.	50	4–21	or.	0/5		0+	−11

[a] i.t. = intratumoral; i.p. = intraperitoneal; or. = oral.
[b] T/C = treated/control.
[c] + significant.
[d] BWC = body weight change.

Table 4. Efficacy of HPC in comparison to its probable metabolites in P388/Mitox

Substance	Dose mg/kg/d	Schedule (day)	Route	T/C[a] (%)	Toxic/total	BWC (%)
HPC	100	0–3	or.	130+	1/10	−16
Hexadecylphosphate sodium	84.5	0–3	or.	65+	5/10	−11
	84.5	0–3	i.p.	30+	10/10	−14
	42.2	0–3	or.	110	3/10	−9
	42.2	0–3	i.p.	30+	10/10	−9
Hexadecanol	59.5	0–3	or.	100	0/10	−5
	59.5	0–3	i.p.	100	0/10	−9
	30	0–3	or.	105	0/10	−4
	30	0–3	i.p.	115	0/10	−2
Dicetylphosphate	134	0–3	or.	110	0/10	−3
	134	0–3	i.p.	85	3/10	−7
	67	0–3	or.	100	0/10	0
	67	0–3	i.p.	90	0/10	−7

[a] + significant.
Abbreviations: see Table 3.

experiments between dose and antineoplastic efficacy or toxicity. The results in the human xenotransplanted mammary carcinoma MT-1 were especially impressive. An oral treatment over three weeks resulted in complete remission of all tumors tested. Similar results were also obtained in this model with the two other etherlipids OPC and EPC (Table 5).

The most interesting results were observed in the mitoxantrone-resistant P388 (P388/Mitox) tumor. In this model, HPC caused a significant prolongation of survival time after both i.p. and oral use; this effect was relatively independent of dose. In the parent P388, HPC had no antineoplastic efficacy. As we knew that the P388/Mitox has a higher immunogenicity than the original line, we wondered whether HPC acts via an immunological mechanism. However, in an experiment using HPC in a prophylactic manner both in immunized (with irradiated P388/Mitox cells) or non-immunized animals no effect could be observed (data not shown).

We were interested in whether the probable metabolites of HPC, hexadecylphosphate-Na, hexadecanol or dicetylphosphate, could be responsible for the antitumor effects of HPC. In an experiment in the P388/Mitox, only HPC was effective; none of the metabolites used in equimolar doses were (Table 4).

Table 5 documents the results of several experiments investigating the antineoplastic activity of OPC and EPC. These compounds had no efficacy in the four murine tumor models used, but were active in the human MT-1 mammary carcinoma.

Results of an experiment using alkylphospholipids as constituents of liposomes can be seen in Table 6. We chose mitoxantrone as encapsulated cytostatic drug, as we have experience with liposomal preparations containing hydrogenated egg phosphatidylcholine (HEPC), especially in the P388 leukemia. All liposomal preparations had almost the same antineoplastic efficacy and toxicity as the free substance. Empty liposomes did not influence tumor growth or toxic parameters. Interestingly, leukopenia caused by mitoxantrone was expressed more when using etherlipid liposomes compared to the free drug and to HEPC liposomes.

As lipids, and especially etherlipids, are known to cause hemolysis of erythrocytes, we tested the capacity of HPC, OPC and EPC following the i.v. administration of doses which were also used in therapeutic experiments (Table 7). While OPC and EPC caused toxic deaths, this was not observed with HPC. Also, the hemolytic activity of both these substances was distinctly higher than that of HPC. In addition, the mice showed hematuria, and we presume that this also contributes to the toxic deaths in other experiments. Following oral administration no hemolysis was ever observed, but cannot be excluded totally.

Discussion

The alkylphosphocholine HPC and its analogs are interesting etherlipids with a simple structure. These chemicals exhibit cytostatic activity against a number of tumor cell types *in vitro* and against a chemically induced rat mammary carcinoma *in vivo*.[1-3]

Table 5. Antineoplastic effect of OPC and EPC in different murine tumor models

Tumor model	Substance	Dose (mg/kg/d)	Schedule (day)	Route	Toxic/total	Survival T/C (%)	Tumor growth T/C(%)	BWC (%)
P388 i.p.	OPC	200	1	i.p.	5/6	25+		−14
		100	1	i.p.	0/6	95		7
		10	1	i.p.	0/6	90		11
	EPC	200	1	i.p.	6/6	30+		—
		100	1	i.p.	0/6	100		−3
		10	1	i.p.	0/6	100		10
	OPC	100	1–4	i.p.	6/6	40+		−13
		50	1–4	i.p.	0/6	110		1
		25	1–4	i.p.	0/6	100		6
	EPC	100	1–4	i.p.	6/6	45+		−11
		50	1–4	i.p.	3/6	80+		−11
		25	1–4	i.p.	0/6	100		2
B 16 i.m.	OPC	50	1–4, 7–11	i.p.	10/10	24+	—	−11
		25	1–4, 7–11	i.p.	2/10	97	93	−6
		12, 5	1–4, 7–11	i.p.	0/10	89	104	−3
	EPC	50	1–4, 7–11	i.p.	1/10	102	91	−7
		25	1–4, 7–11	i.p.	0/10	97	91	−7
		12, 5	1–4, 7–11	i.p.	0/10	98	93	1
P388/Mitox i.p.	OPC	25	1–4	or.	0/9	109		6
		25	1–4	i.p.	0/10	100		8
	EPC	25	1–4	or.	0/8	100		3
		25	1–4	i.p.	0/10	100		5
C3H Mamma i.m.	OPC	50	11–15, 18–22	or.	3/10		64	5
	EPC	50	11–15	or.	1/10		78	3
MT-1 Mamma s.c.	OPC	50	4–21	or.	0/5		0+	−13
	EPC	50	4–21	or.	0/5		13+	−7

Abbreviations: see Table 3.

Table 6. Alkylphosphocholines as constituents of mitoxantrone-containing liposomes; antineoplastic efficacy in P388 leukemia. Tumor cells were inoculated i.p. on day 0. Treatment followed once i.v. on day 1. Body weight change (BWC) and white blood cells (WBC) were determined on day 4

Treatment	Liposomes	Dose (mg/kg)	T/C (%)	WBC ($\times 10^9$/l)	BWC (%)
Mitoxantrone in SUV[a]	HEPC: CH: DCP 1: 1: 0, 25	5	195+	2.2	−7
Mitoxantrone in SUV	EPC: CH: DCP 1: 1: 0, 25	5	175+	1.7	−8
Mitoxantrone in SUV	OPC: CH: DCP 1: 1: 0, 25	5	170+	1.5	−9
Mitoxantrone in SUV	HPC: CH: DCP	5	175+	1.5	−5
Empty SUV	HEPC: CH: DCP	—	100	5.5	1
Empty SUV	EPC: CH: DCP	—	105	5.7	1
Empty SUV	OPC: CH: DCP	—	100	5.9	4
Empty SUV	HPC: CH: DCP	—	100	5.9	4
Mitoxantrone	—	5	170+	2.0	−3
Saline	—			5.6	3

[a] SUV = small unilamellar vesicles.

Table 7. Hemolytic capacity of etherlipids 30 min following intravenous injection

Substance	mg/kg	Toxic/total	Extinction
HPC	100	0/5	0.185
HPC	50	0/5	0.079
OPC	100	5/5	—
OPC	50	0/5	0.154
EPC	100	2/5	1.220
EPC	50	0/5	0.201
Saline			0.062

In addition to previous experiments on MNU- and DMBA-induced mammary tumors of the rat, we here studied the activity of HPC and its analogs with a longer carbon chain (C_{18} and C_{20}) in murine tumor models. At first, we determined the approximate MTD for the mouse with 100 mg/kg/day (resp. 245 μmol/kg/day) for HPC, and a lower MTD of 25–30 mg/kg/day (resp. 57–43 μmol/kg/day) for OPC and EPC. Compared to the literature, the substances were better tolerated in mice than in rats (MTD 46.4 mg/kg/day).[4]

Early results of Berger *et al.* showed also that increasing the chain length of alkylphospholipid (APL) with a carbon number of 14–18 led to a higher toxicity.[10] The reasons for increasing toxicity *in vivo* may be related to the changed solubility and detergent activity connected with increasing chain length. The consequence of this could be a higher hemolytic effect, especially of OPC and EPC. Unger[1] and Nuhn[11] determined the hemolytic activity *in vitro* for HPC and OPC, which showed lytic activity higher or similar to Et-18-OCH_3. It is strongly related to the chain length.

In agreement with other experiments[4] the therapy in classical tumor models P388 leukemia and the B 16 melanoma failed with all three APLs. While the murine tumor Ca 755 was resistant to our alkylphosphocholines, the first transplant generation of the C3H murine tumor showed a moderate response to HPC. Especially surprising were the complete remissions of the human MT-1 mammary carcinoma in nude mice following treatment with HPC, OPC or EPC. The reason for the promising effect of these substances, especially in human mammary carcinomas, is so far unclear.

The high selective activity of APL may be due to the presence of PAF-related receptors in the cells, as was recently demonstrated for receptor-mediated cell destruction by Et-18-OCH_3.[12] This could also be the cause for the specific activity in two out of several tumor models. In addition or in contrast to the cytotoxicity by enzymatically formed active intermediates, the receptor-mediated activity on tumor cells could become more important.

In vitro experiments[13] showed, that Et-18-OCH_3, Ilmofosine and HPC had no cross-resistance in a colchicine-resistant chinese hamster ovary cell line. By contrast, these substances revealed a modest but significant cross-resistance in two adriamycin-resistant cell lines. The authors derive from these results the expectation that membrane-toxic lipids could play a role in antitumor drug resistance.

Surprisingly, we found a significant therapeutic activity in the mitoxantrone-resistant P388 leukemia in contrast to the results obtained in the parent P388. We suppose that this effect could be due to immunological mechanisms, which will be studied by us later.

Additionally, we examined the therapeutic effect of possible cleavage products from alkylphosphocholines. Hexadecanol and sodium hexadecylphosphate had no activity *in vivo*. We suggest that these parts of APL are not substrates for the decisive enzymes that produce cytotoxic compounds for alkylysophospholipids. In addition, we studied the possibility of overcoming the unresponsiveness of the P388 leukemia to APL by the use of liposomes. Liposomes are known for their ability to vary the organ distribution and to increase the availability time of an encapsulated drug in the blood circulation.[14] APLs are able to form liposomes because of their amphiphilic properties, but only a few attempts have been made up to now to produce liposomes consisting of etherlipids.[15,16] Our examination of APL-liposomes with encapsulated mitoxantrone showed the same antineoplastic activity as the free drug in the P388 leukemia. Hence further investigations are necessary to determine the therapeutic potency of such drug–APL–liposome systems.

Our results demonstrate the activity of HPC in one murine and one human mammary tumor model in addition to the previously published rat tumors sensitive to HPC. Furthermore, we could show effectivity in a mitoxantrone-resistant P388 leukemia of the mouse following single application of HPC. However, the mechanism of activity is still unclear, and further work has to be done. Our studies show that APLs are very interesting compounds with a relatively uncommon mechanism of activity.

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