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Erucylphosphocholine: pharmacokinetics, biodistribution and CNS-accumulation in the rat after intravenous administration

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Abstract The clinical use of alkylphosphocholines (APC) in cancer patients is restricted because of the high gastrointestinal toxicity and the need for oral administration. Therefore we evaluated the clinical pharmacology of erucylphosphocholine (ErPC), the first derivative of the APC family suitable for intravenous administration with strong antineoplastic activity, in vitro and in vivo in rats. The pharmacokinetic parameters after a single intravenous dose of 40 mg/kg were calculated using a two-compartment model: $C_{max} = 1.6 \pm 0.3$ μmol/ml, $T_{1/2\alpha} = 0.18 \pm 0.09$ h, $T_{1/2\beta} = 3.3 \pm 0.88$ h, clearance $= 9.7 \pm 1.2$ ml/h, AUC $= 2.5 \pm 0.3$ μmol/ml per h and Vss $= 40.4 \pm 7.9$ ml. Biodistribution studies were performed after repeated ErPC administration at different doses. Intravenous injections of 20 mg/kg given at intervals of 48 h for up to 4 weeks were well tolerated. Neither clinical evaluation nor laboratory parameters (haematology and clinical chemistry) revealed toxic side effects. In contrast, higher doses of ErPC (40 mg/kg per 48 h) led to weight loss. After 2 and 4 weeks of therapy with 20 mg/kg per 48 h a high ErPC accumulation was found in the adrenal glands, small intestine and brain. The brain to serum concentration ratios averaged 2.1 after 2 weeks and 4.5 after 4 weeks. Significant leucocytosis and thrombocytosis were observed after 4 weeks of ErPC treatment. The findings suggest that ErPC is a suitable candidate for clinical trials. In particular, owing to the high accumulation in brain tissue, ErPC is a potential agent for chemotherapy against malignant brain tumours.

Key words Alkylphosphocholines · Erucylphosphocholine (ErPC) · Biodistribution · Pharmacokinetics · Chemotherapy

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Introduction

Alkylphosphocholines (APC) are a new class of synthetic ether lipids with potent antineoplastic activity. In contrast to the alkyllysophospholipid derivatives such as 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine ($Et18OCH_3$) which show rapid metabolism in vivo [2, 21], the APC have shown a high bioavailability in animal experiments [22]. However, in spite of their strong antineoplastic activity against various tumour cell lines in vitro [12, 24, 26] and a proven antitumour effect in several animal models [7, 14, 25], there has been little progress in the last few years in clinical evaluation of their efficacy in cancer patients. The use of the first drug introduced into anticancer therapy (hexadecylphosphocholine, HePC, Miltex™) has been limited by its untoward side effects. In aqueous solutions HePC forms micellar structures with critical micellar concentrations comparable to those of lysolecithins. Following intravenous injections severe haemolysis is observed [19]. Additionally necrosis and thrombophlebitis occur at the injection site [17]. In liposomal formulations, HePC produces no haemolytic effects or local toxicity [9, 17] but is eliminated rapidly from the circulation [18]. The high gastrointestinal toxicity is the dose-limiting side effect after oral administration and occurs even at ineffective doses [5, 23, 29]. Because of these therapeutic difficulties HePC has only been approved for topical treatment of skin metastases in breast cancer patients.

Erucylphosphocholine (ErPC), a new analogue of HePC, shows strong antineoplastic activity against

autochthonous methylnitrosourea-induced mammary carcinomas in rats [6, 20] and brain tumour cell lines in vitro [11, 16]. ErPC is more lipophilic than HePC (Fig. 1), forms lamellar structures and exhibits no haemolytic effects [9]. Therefore, ErPC can be injected intravenously. Comparison of oral treatment with ErPC and HePC in rats bearing MNU-induced mammary carcinomas has revealed a greater accumulation of ErPC in tumour tissue [20]. Considering ErPC the most suitable derivative of alkylphosphocholines for anticancer therapy, the aim of this study was to evaluate the pharmacology and toxicology of ErPC after systemic administration to healthy rats.

Materials and methods

Chemicals

ErPC (relative molecular mass 489.70 Da) was synthesized by H. Eibl. Erucic acid was purchased from Fluka (Buchs, Switzerland), and was 94.3% pure as determined by gas chromatography. Recrystallization of this material from hexane at −4 °C improved the purity to 99.2%. Erucic acid was reduced to erucanol with $LiAlH_4$ in tetrahydrofuran. The erucanol (purity 99.4%) was then converted to ErPC in a two-step procedure. Step 1 comprised three chemical reactions: phosphorylation of erucanol with phosphorus oxychloride, ring closure of the respective erucylphosphorus dichloride with *N*-methyl-ethanolamine, and selective opening of the oxazaphospholane ring at the P–N bond using slightly acidic conditions to form erucyl-phospho-(*N*-methyl)-ethanolamine. Permethylation of the intermediate phospho-(*N*-methyl)-ethanolamine was achieved in step 2, using dimethylsulphate in the presence of potassium carbonate. The chemical structure of ErPC is shown in Fig. 1.

For aqueous solutions, ErPC was dissolved in a mixture of distilled water and ethanol (94:6 v/v) to a final concentration of 8 mg/ml ErPC and stored at 4 °C. For intravenous injection the ErPC solution was heated to 37 °C and then subjected to sterile filtration (Minisart 0.2 μm, Sartorius, Göttingen, Germany).

Animals

Male Wistar rats weighing between 230 and 305 g were used. Animals were kept under conventional controlled conditions and had free access to a standard diet (Altromin) and tap-water during the whole experimental period.

Treatment groups

Pharmacokinetic studies

Four rats (300–305 g) received a single bolus injection of ErPC at a dose of 40 mg/kg body weight (b.w.) via a central venous catheter.

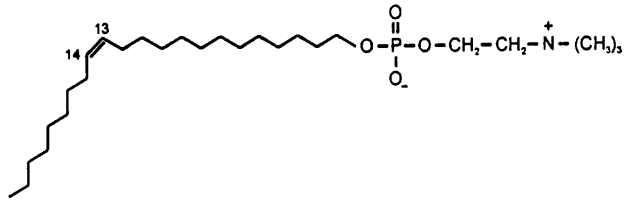


Fig. 1 Chemical structure of erucylphosphocholine (ErPC; relative molecular mass 489.7 Da)

Biodistribution and toxicity studies

A group of 23 rats were treated by repeated intravenous administration of ErPC. Equivalent doses of 10 or 20 mg/kg b.w. per day were given to the conscious rats by slow injection via a central venous catheter. The dosing intervals were 48 and 96 h. Four experimental groups were formed: group 1 ($n = 6$) and group 2 ($n = 6$) received 14 days treatment with 20 and 40 mg/kg ErPC, respectively, every 48 h, and group 3 ($n = 6$) and group 4 ($n = 5$) received 28 days treatment with 20 mg/kg ErPC every 48 h and 40 mg/kg ErPC every 96 h, respectively.

Control group

A group of 39 untreated healthy male Wistar rats used in other experiments weighing 290 ± 40 g were selected to derive normal values of clinical chemical parameters.

Catheters

Silicone catheters (Silastic, Dow Corning Corporation; Midland, Mich.; i.d. 0.51 mm, o.d. 0.94 mm) were prepared as described by Harms and Ojeda [13]. The tubes were filled with heparinized normal saline and the end was sealed with a 24 gauge pin. Rats were anaesthetized by intraperitoneal pentobarbital (50 mg/kg). The external jugular vein was cannulated, the tip of the catheter was advanced to the vena cava superior and the catheter was fixed by a fine suture. After testing for correct function, the tube was passed subcutaneously to the back of the neck, the skin was pierced and the catheter fixed by tight suturing of the small cutaneous incision.

Removal of tissue fluids and organ specimens

In the pharmacokinetic studies blood samples (350–450 μl) were taken using heparinized tubes before and 0.05, 0.5, 1, 2, 3, 4, 7.5, 12.5, 22 and 31.75 h after bolus injection of ErPC for determination of plasma concentrations. The samples were centrifuged at 4000 r.p.m. and frozen at −20 °C until analysis. For detection of the free nonprotein-bound ErPC fraction plasma samples were filtered using a Diaflo™ 10 YM ultrafiltration system with a cut-off at a molecular size of 10,000 Da (Amicon, Danvers, Mass.). In the biodistribution studies, blood samples were taken before (400 μl) and throughout the experiments (250 μl, up to twice a week) via the jugular catheter for determination of haematological and clinical chemistry parameters and the plasma concentrations of ErPC. At the end of the treatment period (day 14 or 28) rats were anaesthetized by intravenous pentobarbital (30 mg/kg) and heparinized by a bolus dose of 300 U/kg. After making a dorsal neck incision, the cisterna magna was punctured using a 28 gauge needle and 150 μl cerebrospinal fluid was withdrawn. The animals were then placed in a supine position and the bladder punctured for urine collection. The left ventricle was cannulated and blood samples were withdrawn (EDTA blood and serum). Then the right atrium was incised, arterial perfusion with 100 ml 0.9% NaCl was performed and organ specimens were removed (mesenteral fat, liver, spleen, small intestine, colon, stomach, kidney, adrenal glands, muscle, heart, lung, brain and skin) and stored at −20 °C until analysis.

Analysis of ErPC in body fluids and tissues

ErPC concentrations were measured by HPTLC as previously described [22]. Organs were minced and homogenized before analysis. Lipid extraction with chloroform/methanol (2:1 v/v) was repeated twice. After drying the organic extracts under nitrogen, lipids were redissolved in chloroform/methanol/water (30:60:8 v/v/v) and applied to HPTLC plates (silica gel 60, Merck Darmstadt, Germany). The plates were developed in chloroform/methanol/triethylamine/

water (30:35:34:8 v/v/v/v), dried at 180 °C, stained with a solution of $CuSO_4$ (10%) in H_3PO_4 (8%) and quantified by densitometry (CD60, Desaga Heidelberg, Germany). The lower detection limit of this method was 10–20 nmol/ml.

Haematological and clinical chemical parameters

The haematological and serum parameters were measured twice before starting and at the end of the treatment period. Blood cells (erythrocytes, leucocytes and thrombocytes) were counted using a haemocytometer (Minos STE, ABX, Göppingen, Germany). The haemoglobin concentration was determined photometrically. The haematocrit of the blood samples was measured after centrifugation in a Heraeus Christ microfuge at 14,000 r.p.m. Serum parameters (Na, K, Ca, glucose, protein, aminotransferases GOT and GPT, lactate dehydrogenase, bilirubin and creatinine) were analysed using a Beckman autoanalyser (Synchron CX5D, Beckman, München, Germany).

Statistical evaluation

For statistical analysis of the data Student's *t*-test was used. Mean values ± SD are presented unless otherwise indicated.

Results

Pharmacokinetics after single bolus injection

The single bolus injection of 40 mg/kg b.w. ErPC (81.7 µmol/kg) resulted in a mean peak plasma concentration of 1.6 ± 0.29 µmol/ml. The plasma concentration decreased with an initial half-life of 0.18 ± 0.09 h and a terminal half-life of 3.3 ± 0.88 h (Fig. 2a–d). The pharmacokinetic data obtained after a single intravenous dose of ErPC were calculated according to a two-compartment model and are shown in Table 1. Only low concentrations of ErPC were found in the organs after a single administration (data not shown). This was in part caused by the high percentage of plasma protein-bound drug. No free ErPC was detectable in plasma ultrafiltrate 5 min after the bolus injection.

Biodistribution after repeated intravenous injections

The repeated intravenous administration of ErPC resulted in a dose- and time-dependent increase in serum concentration. After 4 weeks of treatment the serum concentrations had not yet reached steady-state conditions (Fig. 3). The serum levels averaged 65.5 ± 27.7 nmol/ml and 88.5 ± 19.9 nmol/ml in group 1 (20 mg/kg per 48 h) and 2 (40 mg/kg per 48 h), respectively, after 14 days of ErPC treatment and 64.5 ± 18.0 nmol/ml and 51.7 ± 11.8 nmol/ml in group 3 and 4 (20 mg/kg per 48 h and 40 mg/kg per 96 h), respectively, after 28 days of ErPC treatment. The biodistribution of ErPC after 2 and 4 weeks of treatment is shown in Fig. 4. A particularly high accumulation of ErPC was found in the adrenal glands, small intestine and, interestingly, in the brain tissue while low concentrations were found in colon, stomach, muscle and fat tissue. No ErPC was detected in the cerebrospinal fluid

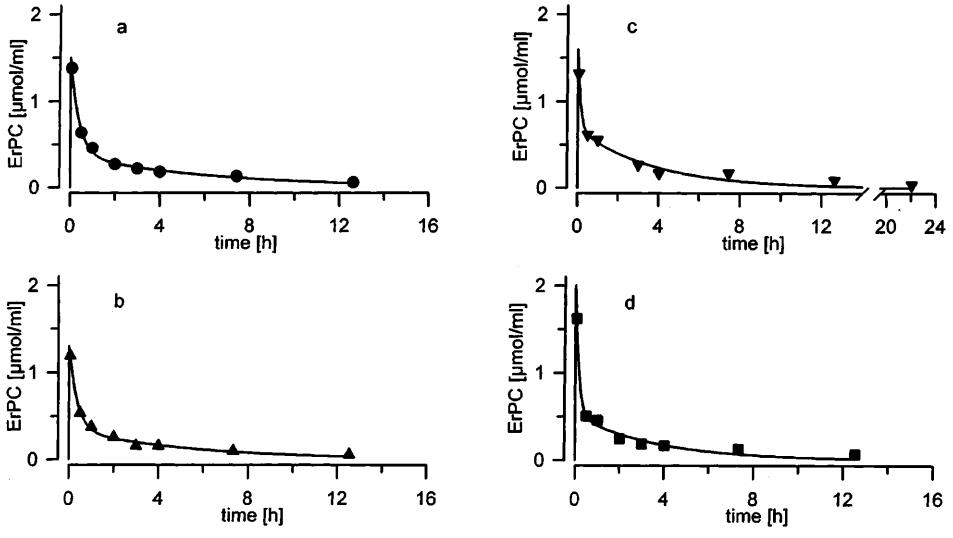


Fig. 2a–d Plasma concentration curve of ErPC after a single intravenous injection of 40 mg/kg. Four individual experiments are shown (**a–d**)

Table 1 Pharmacokinetic parameters obtained for ErPC in plasma after a single intravenous injection of 40 mg/kg body weight (two-compartment model) (C_{max} maximal concentration of ErPC, $T_{1/2\alpha}$ initial half-life, $T_{1/2\beta}$ terminal half-life, *CL* total body clearance, *AUC* area under the concentration-time curve, V_{SS} volume of distribution)

	Dose (mg)	C_{max} (µmol/ml)	$T_{1/2\alpha}$ (h)	$T_{1/2\beta}$ (h)	CL (ml/min)	AUC (µmol/ml · h)	V_{SS} (ml)
Mean	11.6	1.6	0.18	3.30	9.55	2.46	39.68
SE	±0.16	±0.29	±0.09	±0.88	±1.13	±0.32	±7.75

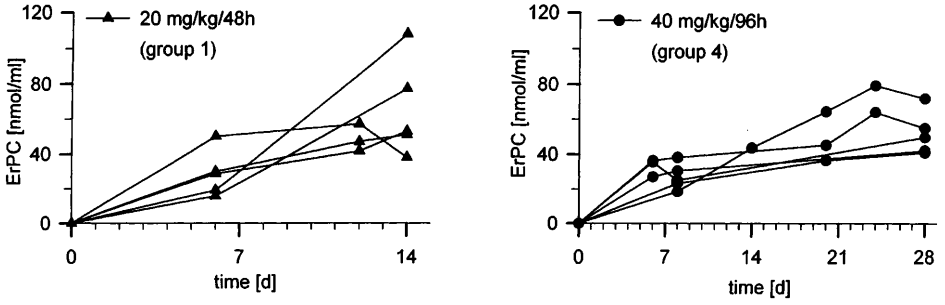


Fig. 3 Serum concentrations of ErPC after repeated intravenous administration of 20 mg/kg at intervals of 48 h (*left side*, group 1) and of 40 mg/kg at intervals of 96 h (*right side*, group 4)

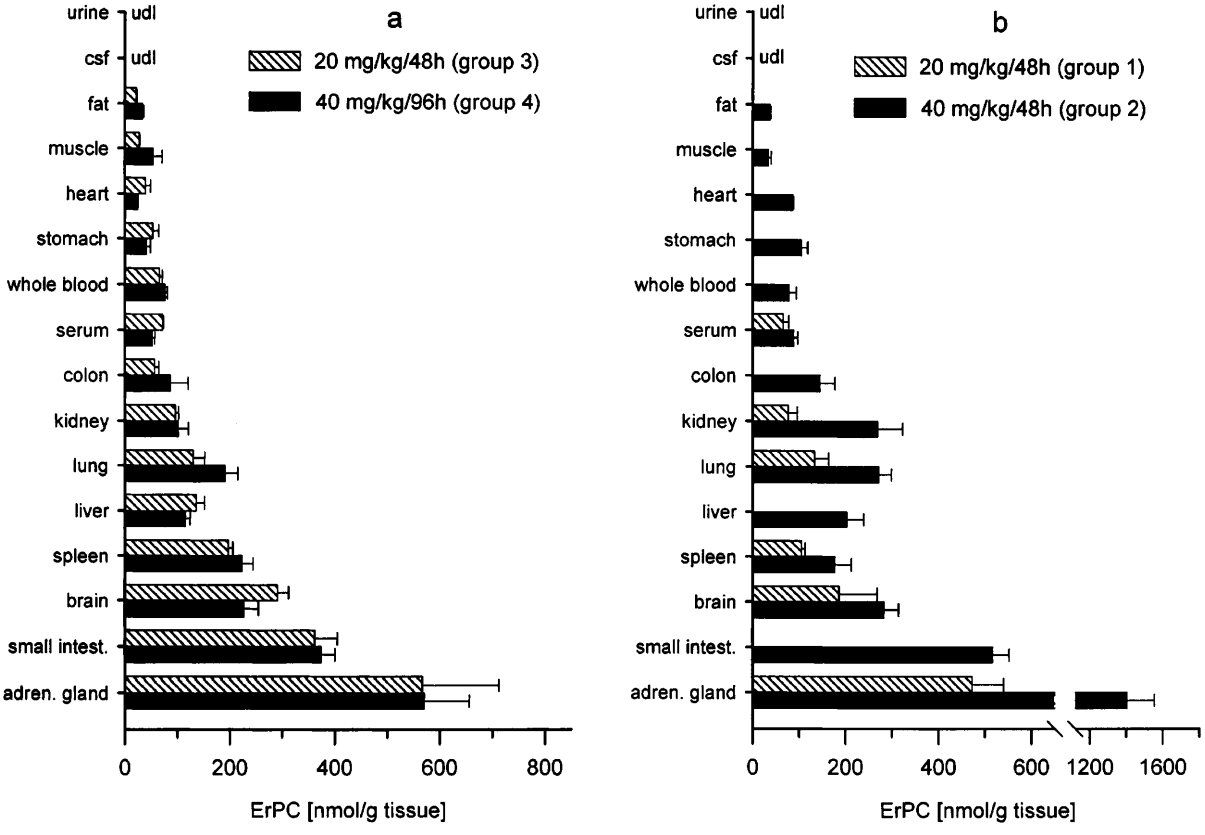
or urine. The enrichment of ErPC in brain tissue was time- and dose-dependent (Fig. 5).

Doubling the dose (group 2) or the treatment period (group 3) led to significantly higher ErPC levels in the brain than treatment at the 20 mg/kg dose level over 14 days (group 1; $P < 0.05$). The brain to serum concentration ratio averaged between 2.1 in group 1 and 4.5 in group 4. No differences in ErPC concentrations or biodistribution pattern were found between the 48-h dosing interval and the 96-h interval at the equivalent dose of 10 mg/kg per day (group 4; Fig. 4a). In contrast, the higher dose of 40 mg/kg given every 48 h (group 2) resulted in considerably higher serum and organ concentrations.

Toxicity and side effects

The long-term treatment of rats over a period of 2 or 4 weeks by repeated intravenous bolus injections of ErPC at intervals of 2 or 4 days at an equivalent dose of 10 mg/kg b.w. per day was well tolerated and caused no clinical side effects. A thorough daily examination of the physical state of the animals did not reveal any neurological symptoms or behavioural disturbances. The good general condition of the animals was documented by a continuous normal weight gain (Fig. 6). However, the higher dose of 20 mg/kg per day (40 mg/kg per 48 h; group 2) resulted in a loss of body weight after 6 days of ErPC therapy probably due to loss of appetite or nausea

Fig. 4a,b Biodistribution of ErPC after repeated intravenous injections. The concentrations of ErPC in different organs and body fluids after intravenous administration over 2 and 4 weeks are shown. **a** Treatment for 4 weeks with 20 mg/kg every 48 h and with 40 mg/kg every 96 h; **b** treatment for 2 weeks with 20 mg/kg every 48 h and 40 mg/kg every 48 h. Values are means ± SEM (*udl* value under detection limit). *Note*: In group 1 not all organs could be analysed



(Fig. 6, upper part), and was considered to be the maximum tolerated dose. In general, at the end of the treatment course there were no macroscopic signs of organ injury in any group. Table 2 provides an overview of the serum parameters during ErPC treatment. There were no changes in clinical chemistry values at the end of ErPC treatment in groups 1, 3 and 4 from the values obtained on day 0 and no differences compared with the values obtained from normal untreated control rats. Slightly higher serum GOT and GPT activities and a higher creatinine concentration were found at the end of the treatment period in group 2.

Regarding the haematological parameters, no signs of bone marrow toxicity could be detected. In contrast, the white blood count increased during ErPC treatment. After 4 weeks of treatment a significant leucocytosis was observed. The number of leucocytes increased from 8170 ± 150 to 15,550 ± 3430/µl in group 3 and from 8940 ± 1400 to 17,240 ± 4520/µl in group 4 ($P < 0.05$). The platelet count also showed a significant increase in groups 2 and 4 (623 ± 144/nl to 973 ± 89/nl and 520 ± 33/nl to 702 ± 67/nl, respectively, $P < 0.05$). The slight thrombocytosis in groups 1 and 3 was not statistically significant. Red blood cell count, haemoglobin concentration and haematocrit showed no alterations during ErPC treatment.

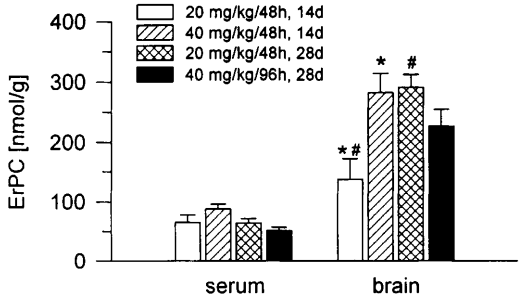


Fig. 5 Concentrations of ErPC in serum and brain tissue after repeated intravenous ErPC injections over 2 and 4 weeks. There is a high enrichment of ErPC in brain. Significant differences were also observed in the brain ErPC concentrations. Values shown are means ± SEM, $*^{\#}P < 0.05$ (Student's *t*-test)

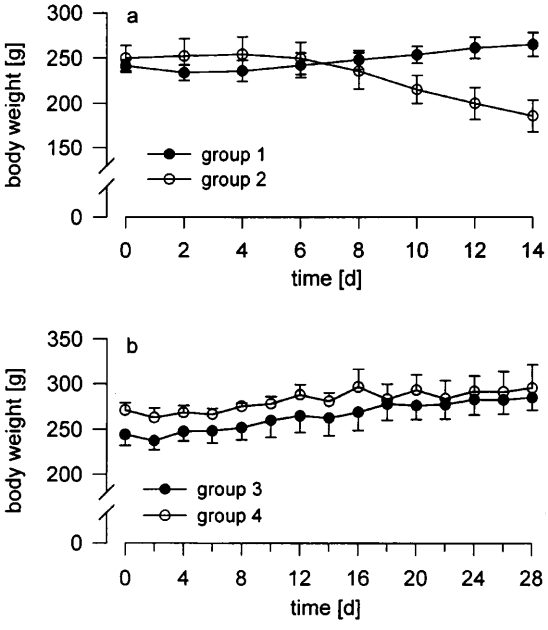


Fig. 6a,b Change in body weight of animals treated with intravenous ErPC over 2 weeks (**a**) and 4 weeks (**b**). Values shown are means ± SD. Note the weight loss of the rats during the second week of treatment with 40 mg/kg per 48 h (*open circles* in the upper part)

Discussion

During the last two decades ether lipids have been shown to exhibit strong antineoplastic effects in vitro and in vivo [1, 3, 10]. Up to now the clinical use of this

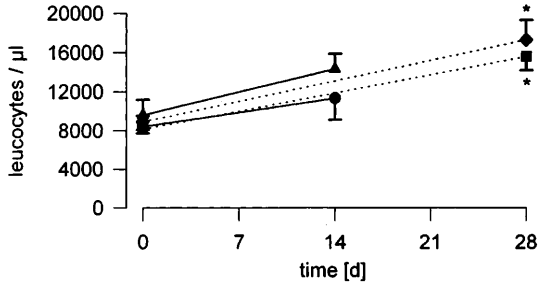


Fig. 7 Increase in white blood cell count after 2 and 4 weeks of ErPC treatment. The leucocyte numbers after 4 weeks (groups 3 and 4) are significantly higher than the baseline values before ErPC therapy (▲ group 1, ● group 2, ■ group 3, ◆ group 4). $*P < 0.05$, Student's *t*-test

Table 2 Serum parameters during ErPC treatment

Treatment group	Day	Na (mmol/l)	K (mmol/l)	Ca (mg/dl)	Glucose (mg/dl)	Protein (g/dl)	GOT (U/l)	GPT (U/l)	LDH (U/l)	Creatinine (mg/dl)
Control		142 ± 3	4.4 ± 0.5	10.2 ± 0.7	152 ± 14	5.1 ± 0.4	33 ± 9	35 ± 9	229 ± 135	0.47 ± 0.11
1	0	143 ± 1	5.0 ± 0.4	9.9 ± 0.7	129 ± 7	5.5 ± 0.3	44 ± 8	44 ± 13	434 ± 265	0.35 ± 0.10
	14	141 ± 2	4.8 ± 0.3	9.6 ± 0.6	142 ± 17	4.7 ± 0.3	37 ± 6	40 ± 6	348 ± 208	0.42 ± 0.03
2	0	143 ± 0	5.6 ± 0.3	10.4 ± 0.9	155 ± 48	5.8 ± 0.7	30 ± 1	46 ± 11	181 ± 33	0.27 ± 0.01
	14	143 ± 3	4.5 ± 0.7	9.6 ± 0.6	127 ± 86	4.2 ± 0.3	62 ± 19	60 ± 17	339 ± 148	0.63 ± 0.10
3	0	139 ± 5	4.5 ± 0.5	10.5 ± 0.4	133 ± 3	5.3 ± 0.3	38 ± 5	33 ± 4	244 ± 155	0.46 ± 0.04
	28	141 ± 2	4.8 ± 0.5	9.4 ± 0.6	135 ± 19	5.2 ± 0.2	45 ± 5	32 ± 6	581 ± 538	0.40 ± 0.05
4	0	142 ± 3	5.9 ± 0.7	9.8 ± 0.5	130 ± 14	5.6 ± 0.4	40 ± 14	45 ± 11	539 ± 243	0.36 ± 0.09
	28	141 ± 2	4.8 ± 0.3	9.7 ± 0.8	136 ± 14	5.0 ± 0.3	36 ± 2	34 ± 4	332 ± 138	0.41 ± 0.08

new drug family in anticancer therapy has been restricted by the lack of a derivative suitable for intravenous administration. With oral administration of HePC, the prototypical APC, the tolerated doses in clinical studies have been too low to achieve plasma levels sufficient for cytotoxic activity [5, 23, 29]. The gastrointestinal toxicity occurring even at low doses has limited its therapeutic use. Thus, there has been little progress in the clinical evaluation of the efficacy of HePC – and the APC in general – in cancer patients.

ErPC is the first analogue of APC suitable for parenteral administration. After a single intravenous injection of ErPC only low concentrations were found in the organs while repeated administrations resulted in a continuous increase in serum ErPC concentrations and a high accumulation of ErPC in several organs (e.g. adrenal glands, small intestine, brain, spleen and lung). The continuous rise in the serum levels after repeated ErPC injections suggests that there is an additional terminal half-time of the drug much longer than 3.3 h, which could not be determined after a single injection. The high percentage of plasma protein-bound drug reflects the serum distribution of HePC, which is also almost completely bound to serum albumin [19].

The biodistribution patterns determined in this work confirm the results of Kötting et al. [20], who described a similar organ distribution after oral ErPC administration. However, the biodistribution of ErPC clearly differed from the organ distribution of orally administered HePC. The highest tissue concentrations of HePC were found in the kidneys [22] which is perhaps the reason for the renal dysfunction observed in patients receiving oral HePC in clinical phase I and II studies [23, 29]. Compared to HePC, ErPC showed only a weak enrichment in renal tissue but a surprisingly strong accumulation was found in the brain. Such high concentrations in the CNS have not been reported with any other ether lipid exhibiting anticancer properties. On the other hand, during oral HePC treatment, a continuous increase in the drug concentration in brain tissue has also been found, albeit at very low levels [8].

There are only few reports available comparing the antitumour effects of HePC and ErPC. After oral treatment over 11 days ErPC showed a higher accumulation in the tumour tissue of methylnitrosourea-induced mammary carcinomas in rats compared to HePC, suggesting a superiority of ErPC in cancer therapy [20]. The distinct accumulation of ErPC in brain tissue is of particular interest. Disappointing results of nearly all clinical studies so far using chemotherapeutic agents in the treatment of malignant brain tumours justify the search for new substances with antitumour activity against brain tumours. Berdel et al. have reported a cytotoxic effect of alkyl lysophospholipids in human brain tumour cells [4]. In vitro experiments in our laboratory have revealed time- and concentration-dependent cytostatic and cytotoxic effects of ErPC on a rat and several human brain tumour cell lines. At concentrations between 70 and 110 μM complete cell death was induced within 48–96 h of incubation. Additionally it has been shown that apoptosis contributes to the cytotoxicity of ErPC [16]. The cytotoxic effects against different brain tumour cell lines occur at lower concentrations compared to HePC [11, 16]. In the present study the concentrations achieved in the brain tissue greatly exceeded the ErPC concentrations required for in vitro effectiveness. Therefore ErPC is a potential new drug for clinical trials in brain tumour chemotherapy. In vivo experiments in rats bearing intracerebral or subcutaneous C6 tumours have revealed a high enrichment of ErPC in the subcutaneous tumour tissue and a strong concentration-response relationship in peripheral gliomas [11]. Intracerebral C6 gliomas showed a less-marked response to ErPC in these experiments probably because of a treatment period too short to achieve significant tumour reduction.

No organ toxicity was found using ErPC doses able to achieve cytotoxic concentrations in most of the tissues analysed. The dose-limiting side effect of ErPC preventing further dose escalation was loss of appetite and weight loss. A continuous increase in the number of leucocytes and platelets has also been found in clinical trials using HePC [23, 28] indicating that this effect might be a common feature of APC. The mechanism of this effect is not yet fully understood. While in vitro studies of the effects of different ether lipids on mouse granulocyte-macrophage progenitor cells have revealed a growth inhibition of the haematopoietic precursors, no bone marrow toxicity has been observed in vivo [27]. The leucocytosis induced by HePC has led to the concept of the coadministration of APC and myelotoxic anticancer drugs in order to weaken the toxicity to haematopoietic stem cells [15]. Whether such a regimen is successful has to be confirmed by clinical investigations.

In conclusion, ErPC is the first derivative of the APC family with strong antineoplastic activity in vitro and in animal experiments which can be given by intravenous injection. High tissue concentrations were achieved after treatment over 2 or 4 weeks without toxic side effects. Our findings suggest a superiority of ErPC compared to HePC for clinical use. A particularly high accumulation was found in brain tissue. Thus we can recommend that the anticancer properties of ErPC, particularly against malignant brain tumours, be evaluated in clinical studies.

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