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Original Paper

Induction of Resistance to Hexadecylphosphocholine in the Highly Sensitive Human Epidermoid Tumour Cell Line KB

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Hexadecylphosphocholine (HePC, Miltefosine) is a representative of the group of alkyl-lysophosphocholines showing remarkable antitumoral activity in *in vitro* experiments and in experimental animal tumour models. The epidermoid tumour cell line KB, which is highly sensitive to HePC (half-maximal growth inhibiting concentration, IC_{50}: 1.2 μM; half lethal concentration, LC_{50}: 2.8 μM), was slowly adapted to increasing concentrations of HePC. After 14 months, the adaptation process was stopped at a concentration of 10 μg/ml (23.5 μM). At this point, the KB cells tolerated high doses of HePC (IC_{50}: 41.2 μM; LC_{50}: 87.1 μM). The resistant cells (KBr) also showed crossresistance to the other well studied ether-lysophospholipids, Edelfosine (1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine, OMG-3PC; ET18OCH3) and Ilmofosine (1 S-hexadecyl-2-methoxymethyl-rac-(1-thio-3-hydroxy)propyl-3-phosphocholine, BM 41.440). Comparison of the KB and KBr cells showed that total lipid phosphate, ether-lipid content, vinyl-ether-lipid content, protein content as well as cholesterol content were unchanged. Furthermore, no changes were observed in the lipid composition between KB and KBr cells. Uptake of choline was also unchanged in both cells, but the uptake of D-myo-inositol was lower by a factor of two in the KBr cells. However, in KB cells, the addition of HePC induced a 50% reduction of D-myo-inositol-uptake, whereas in KBr cells inositol uptake was unchanged. Differences in HePC uptake and HePC metabolism were apparent between the KB and KBr cell lines. KBr cells showed a 3-fold lower uptake for HePC and a 3- to 4-fold faster metabolism of HePC than KB cells. However, the amount of non-metabolised HePC after 2 days of incubation with 1 μg/ml HePC (LC_{50}: 1.2 μg/ml) in KB cells was 3- to 4-fold lower than the amount of HePC in KBR cells at 10 μg/ml (LC_{50}: 37 μg/ml), indicating that KBr cells can incorporate higher amounts of HePC than KB cells without adverse effects for cell growth and viability. This seems to indicate that mechanisms other than slower uptake and faster metabolism are involved in the induction of resistance to HePC in KBr cells.

Key words: ether-lysophospholipids, hexadecylphosphocoline, Miltefosine, Miltefosine-resistance
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INTRODUCTION

THE ALKYL-LYSOPHOSPHOLIPID analogue hexadecylphosphocholine (HePC, Miltefosine) is a representative of the group of alkylphosphocholines [1]. It is cytotoxic for a variety of tumour cell lines in culture [2, 3]. Oral treatment of tumour bearing animals with HePC results in significant antitumoral activity [4, 5]. However, the effects are highly specific for certain tumour types. Autochthonous, carcinogen-induced rat tumours as well as some human tumour cell lines transplanted into nude mice are extremely sensitive, whereas classical murine screening models such as P388 and L1210 are completely insensitive [4].

Early clinical trials with a topical application of HePC in cutaneous metastases of patients with mammary carcinomas have already indicated that this compound is also active in human cancer [6, 7].

The molecular mechanism of action of HePC is still unknown. Studies on the metabolism of HePC in the human leukaemia cell line Raji revealed that HePC is only slowly degraded, with more than 90% of the radiolabel still associated with the parent compound after 3 days of incubation [2].

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Generation of toxic metabolites does not seem to be the key event of HePC cytotoxicity since non-degradable analogues of HePC showed similar antiproliferative activities [8]. There are, however, several lines of evidence that suggest that HePC may interfere with signal transduction mechanisms in proliferating cells. In this respect, an inhibition of protein kinase C in bombesin-induced NIH 3T3 fibroblasts and also in phorbol-ester-stimulated HL60 cells has been reported [9, 10]. Recently, we concluded that protein kinase C is not involved in the mechanism of action of HePC in HL60 cells [11]. Heesbeen and associates came to a similar conclusion for OMG-3PC [12].

In interleukin-2-dependent PMNC cultures, a HePC-induced enhancement of interferon-gamma secretion and an increased expression of interleukin-2 receptors could be demonstrated [13]. In U937 cells, HePC stimulates an increase of Histone H_1^0 gene expression, which clearly precedes growth retardation [14]. In addition, it has been demonstrated that low doses of HePC amplify the haematopoietic growth factor dependent proliferation of progenitor cells *in vitro* [15].

Since these effects cannot explain the action of HePC satisfactorily, we decided to induce HePC-resistance in our most sensitive cell line KB. The differences between the HePC-resistant subline KBr and the parent cell line KB could be of great importance for the elucidation of the mechanism of action of HePC, while these differences are based on the induction of HePC-resistance and not due to different, cell origin dependent, gene regulations. Insight into the mechanism of action of HePC may be of value for the development of better, more efficient ether-lysophospholipid analogues for cancer therapy.

MATERIALS AND METHODS

Materials

OMG-3PC was synthesised as described before [16] and was a gift of Dr H. Eibl, Göttingen. HePC was a gift of Asta-Medica AG (Frankfurt, Germany). BM 41.440 was a gift from Boehringer-Mannheim (Mannheim, Germany). [9,10-^{3}H]Hexadecyl-phosphocholine (11.4 Ci/mmol) was a gift of Asta-Medica AG and was diluted to 60 mCi/mmol prior to purification on preparative TLC-plates. The cholesterol determination kit was purchased from Sigma (Munich, Germany). The phosphorous determination kit, according to Eibl and Lands [17], was obtained from Serva (Munich, Germany). [^{14}C] Choline and [^{3}H]-D-myo-inositol were obtained from Amersham (Braunschweig, Germany) or from Dupont-NEN (Frankfurt, Germany). All other chemicals were of commercial grade and used without further purification.

Cell lines

The KB cell line, a human epidermoid cancer cell line, was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, U.S.A.).

Cell culture

The cells were maintained in Click's/RPMI 1640 medium supplemented with 20 mM *N*-[2-hydroxyethyl]-piperazine-*N'*-[3-ethanesulphonic acid] (HEPES), pH 7.3, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM L-glutamine and 10% fetal calf serum (FCS). Cell cultures were incubated at 35°C, 5% CO_2 and 95% humidity. Cultures were assessed for mycoplasma contamination at regular time intervals. For subcultivation or experiments, the adherent cells were detached from the culture flasks by 0.025% trypsin in 20 mM ethylene-diaminetetra-acetate (EDTA) and washed twice in fresh medium.

Induction of resistance to HePC

KB cells were slowly adapted to HePC, starting with a concentration of 0.01 μg/ml (23.5 nM). When the cells showed normal growth and high viability (> 95%), the concentration of HePC was raised, beginning with steps of 0.01 μg/ml. When higher concentrations of HePC were tolerated, these increments in HePC concentration were raised accordingly (see Figure 1). After a period of 14 months, the HePC concentration reached 10 μg/ml (23.5 μM), at which concentration the adaptation to HePC was stopped. The resistant cells were routinely grown in 10 μg/ml HePC to maintain their tolerance for ether-lysophospholipids.

Toxicity assay

Cells (2×10^5/well) were incubated in six-well plates (3 ml medium/well) with increasing concentrations of HePC, OMG-3PC or BM 41.440 for 48 h at 37°C, 5% CO_2 and 95% humidity. The culture medium was transferred in 15 ml tubes, and 1 ml trypsin/EDTA-solution was given in each well to detach the cells. The detached cells were suspended in the trypsin solution to which 2 ml cell culture medium was added. The cell suspension was added to the corresponding 15 ml tube containing the cell supernatant from the same well and the whole mixture was centrifuged at 400*g* for 10 min. The supernatant was discarded and the cell pellet was resuspended in 1 ml culture medium. Cell number and viability were determined in a Neubauer cell counting chamber by Trypan blue dye exclusion [18]. Half-maximal growth inhibition (IC_{50}) and half-maximal toxicity (LC_{50}) were determined by intrapolation of the values obtained [19].

Phospholipid and cholesterol contents

Cellular lipids were extracted from a known number of cells with chloroform/methanol/water (8/10/4, by v/v/v) according

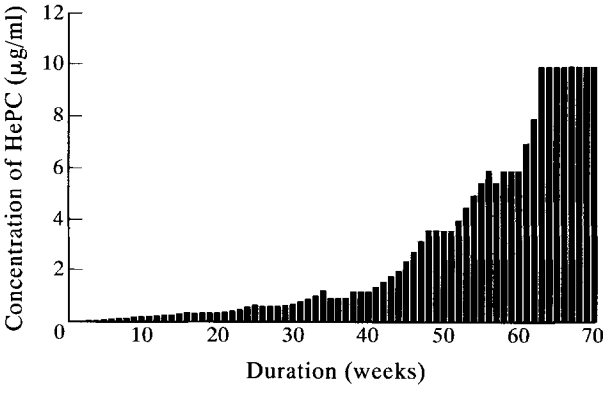

Figure 1. Adaptation of KB cell line to HePC. KB cells were incubated with increasing amounts of HePC, starting at a concentration of 0.01 μg/ml for 1 week. Increments in concentration were made at the end of each week, when cell growth and viability were close to the values for non-treated KB cells. When further increments were not possible, cells were kept at the same concentration for another week or, when cell viability fell below 70%, at a concentration that was one step lower than the actual concentration. Adaptation was stopped after a HePC concentration of 10 μg/ml was reached.

to Bligh and Dyer [20] and dried under nitrogen. These extracts were then redissolved in chloroform/methanol/water (5/10/2, v/v/v), desalted over a Sephadex G-15 column and dried again. Lipid phosphorous was then determined by the method of Eibl and Lands [16]. The amounts of ether- and vinyl-ether-phospholipid were determined from cell extracts that were treated with Na-(tert.)-butylate before lipid extraction, to hydrolyse acyl-phospholipids. For the determination of ether-phospholipids, the cell pellets were also treated with 6 N ice cold HCl to hydrolyse vinyl-ether-lipids before lipid-extraction. Free cholesterol and total cholesterol were assayed, after hydrolysis by an enzymic colour reaction according to Omodeo-Sale and associates [21], using a cholesterol determination kit from Sigma (Munich, Germany).

[^{3}H]-Hexadecylphosphocholine uptake

Cells (0.5 to 4×10^6/ml) were incubated at 37°C for 2 h before [^{3}H]-HePC (0.1 μCi/ml) was added. Simultaneously, unlabelled HePC was given to the desired concentration. At indicated time intervals, aliquots were taken and washed three times in a 10-fold volume of ice-cold PBS containing 5% FCS. Cell pellets were finally lysed in liquid scintillation fluid and cell associated radioactivity was measured by liquid scintillation counting.

Metabolism of HePC

After incubation of the cells with [^{3}H]-HePC at the indicated concentration for 48 h, the cells were harvested, washed three times with 0.9% aqueous NaCl, and counted for cell number and viability. The cells were then pelleted and extracted for phospholipids as described above. Aliquots of the lipid extract were applied to HPTLC plates and the plates were developed in chloroform/methanol/concentrated ammonia (60/40/4, v/v/v). Radioactive bands were detected in a Berthold linear analyser and their radiolabel content was calculated as described previously [19].

Lipid composition

After phosphorus determination, the remainder of the lipid extract was dried under a stream of nitrogen at 60°C. The lipid film was redissolved in chloroform/methanol (1/1, v/v) and applied to HPTLC-thin layer plates (Merck, Darmstadt, Germany). The plates were developed in chloroform/methanol/acetic acid/water (60/50/5/2, by volume) for the analysis of phospholipids or in chloroform/methanol/triethylamine/water (30/34/35/8, by volume) for the separation of lysophosphatidylcholine and HePC, which could not be separated satisfactorily in the phospholipid separation solvent. For a better separation of phosphatidylglycerol and phosphatidic acid, the plates were developed in chloroform/methanol/acetic acid/1 N HCl (200/60/20/5, by volume). After drying, the plates were immersed in an aqueous copper sulphate (10%) solution, made acidic with 8% phosphoric acid. The plates were then charred and the charred spots were quantitated using a personal densitometer (Molecular Dynamics, Krefeld, Germany) as described by Rustenbeck and Lenzen [22] and by Kötting and associates [23].

Protein determination

Cellular proteins were measured using the protein determination kit from Sigma (München, Germany) based on the procedures of Lowry and Peterson [24–26].

Uptake of choline and D-myo-inositol

For uptake of choline or inositol, the cells were incubated in medium depleted of the compound to be studied. The cells were trypsinised and washed cell suspensions were pre-incubated at 37°C in medium either in presence or absence of HePC at the indicated concentration. The temperature was maintained by a thermostat-regulated waterbath. Then, radiolabelled [^{14}C]choline (50 μCi/μmol) or [^{3}H]inositol (80 μCi/μmol) was added. After indicated time intervals, a 500 μl aliquot of the cells was taken and diluted with 5 ml ice cold medium. The cells were centrifuged and the pellet was washed three more times with ice cold medium. Then the cell pellet was taken up in 1 ml distilled water and the suspension was sonicated for 15 min at 4°C. Radioactivity of aliquots of the homogenised suspension was then measured. The uptake per million cells of the compound in question was calculated from the cell number and the known specific radioactivity of choline or inositol.

Statistics

Statistical calculations were performed using the programme INSTAT from Graphpad Software Inc. (San Diego, California, U.S.A.).

RESULTS

Adaptation of KB cells to HePC

For the induction of HePC resistance, the KB cells were grown in gradually increasing concentrations of HePC. Earlier attempts to isolate HePC-resistant KB cells by limiting dilution experiments, or by cloning with exposure to 0.5 μg/ml HePC failed, since no viable cultures could be established. Therefore, we selected the more labour-intensive method of gradual adaptation. At the first concentration, 0.01 μg/ml, the cells did not show any signs of toxic effects and after a week of culture the concentration of HePC could be increased to 0.02 μg/ml. The HePC concentration was raised weekly with steps of 0.01 μg/ml for the first few weeks, until the cells showed a loss in growth rate or in viability, at which stage the concentration was kept constant until the cells had recuperated (Figure 1). When viability decreased below 60%, the concentration was lowered one step and kept there until the cells again showed 95% viability. At later stages, when the HePC concentration to which the cells were adapted was higher, the increment steps were gradually increased and, in the final stage of adaptation, steps of 1–2 μg/ml HePC could be applied to the cell cultures. Throughout the adaptation process, a back-up culture with a concentration that was one step lower than that of the adaptation culture was maintained to avoid a complete loss of HePC-resistant cells, when the adaptation would have been too fast. At a concentration of 10 μg/ml HePC (23.5 μM, almost 10 times LC_{50} of the parent cell line), the adaptation process was stopped and IC_{50} and LC_{50} values were determined. It showed that the cells were able to tolerate approximately 30-fold higher concentrations of HePC and also were approximately 30-fold more resistant to two other ether-lipid analogues OMG-3PC and BM 41.440 (Table 1). The resistant subline (KBr) was used for comparative experiments with the parent KB cell line.

Loss of HePC resistance

The KBr cells lost resistance to HePC when cultured in the absence of HePC for prolonged periods of time (Figure 2). After approximately 12 weeks without HePC, the IC_{50} and

Table 1. Cross-resistance of KBr to OMG-3PC and BM 41.440

Compound	KB		KBr	
	IC_{50}	LC_{50}	IC_{50}	LC_{50}
HePC	0.5 ± 0.1	1.2 ± 0.2	17.5 ± 1.8*	37.0 ± 2.3*
OMG-3PC	0.4 ± 0.1	0.8 ± 0.1	14.0 ± 2.3*	32.2 ± 2.1*
BM 41.440	0.4 ± 0.1	1.4 ± 0.2	9.6 ± 1.8*	28.6 ± 1.2*

IC_{50} and LC_{50} values are in μg/ml ether-lipid analogue. * Difference in IC_{50} or LC_{50}-value, respectively, statistically significant between KB and KBr cells ($P < 0.0005$). Mean and standard deviation, n ranged from eight (OMG-3PC and BM 41.440) to 10 (HePC) independent duplicate experiments.

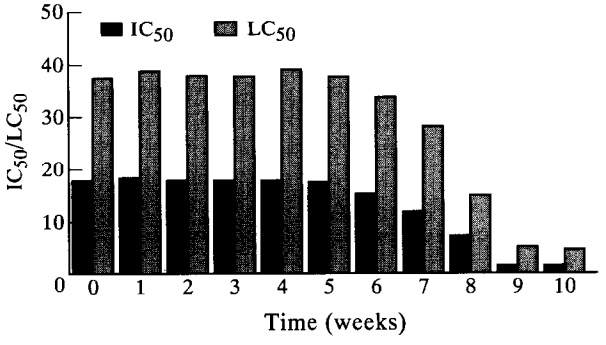

Figure 2. Loss of resistance of KBr for HePC. KBr cells that tolerated 10 μg/ml HePC were cultured in the absence of HePC and at weekly intervals IC_{50} and LC_{50} values were determined. After an initial period of maintaining high tolerance to HePC, the cells showed a loss of resistance to the compound. A lower plateau was reached at IC_{50} and LC_{50} values only twice that of untreated KB cells.

LC_{50} values were only twice that of the parent cell line, indicating that the tolerance of the cells to HePC was not due to a constitutional genetic change.

Comparison of KB and KBr cells

Table 2 shows the data from protein, total phosphorus, lipid phosphorus, ether-phospholipid, vinyl-ether-phospholipid and cholesterol determinations. The values for both cell lines only show a slightly significant change ($P = 0.015$) in the amount of total protein, with the KBr cells having approximately 40% higher values; this may be due to a slightly larger size of KBr cells as observed by microscopy. All other values were not significantly different. Furthermore, the phospholipid composition showed only marginal changes between KB and KBr cells (Table 2).

More pronounced differences were found for the uptake kinetics of D-myo-inositol, whereas the uptake kinetics for choline were similar between both cell lines (Table 3). It showed that the KBr cells were not influenced by HePC in their uptake of D-myo-inositol, with uptake rates half that of the parent cell. KB cells reduced the rate of uptake of D-myo-inositol by 50% in the presence of HePC. KBr cells were cultured in absence of HePC for one week prior to the start of uptake or metabolism experiments to avoid the influence of HePC. For the experiment itself, HePC was added to the KBr cells when the influence of HePC was investigated, identically to the experiments with KB cells.

The uptake and metabolism of HePC were also investigated

Table 2. Comparison of KB and KBr cells with regard to lipid and protein content

	KB	KBr
Lipid phosphate*	62.7 ± 27.5	72.7 ± 30.9
Ether-lipid phosphate*	36.1 ± 25.5	46.9 ± 35.9
Vinyl ether-lipid phosphate*	6.8 ± 6.8	9.9 ± 5.7
Total cholesterol†	7.5 ± 0.6	7.9 ± 0.9
Free cholesterol†	7.2 ± 0.3	7.4 ± 1.0
Total cellular protein†	249 ± 24	363 ± 60
Phospholipids (%) of lipid phosphate:		
Lysophosphatidylcholine and HePC	2.3 ± 0.9	5.6 ± 1.2
Sphingomyelin	12.6 ± 1.0	14.2 ± 2.7
Phosphatidylcholine	35.8 ± 6.5	35.6 ± 5.8
Phosphatidylserine	9.2 ± 1.7	7.9 ± 1.0
Phosphatidylinositol	10.1 ± 1.2	7.9 ± 1.0
Phosphatidylethanolamine	23.5 ± 3.4	24.1 ± 4.1
Phosphatidylglycerol	2.4 ± 0.6	2.4 ± 0.6
Cardiolipin	4.0 ± 0.4	5.6 ± 1.4

Mean and standard deviation from six to eight independent duplicate determinations. None of the values were considered statistically different between KB and KBr ($P > 0.1$), except total cellular protein ($P = 0.015$). * nmol/10^6 cells. † μg/10^6 cells.

Table 3. Uptake of choline and D-myo-inositol and influence of HePC on uptake

	HePC	KB	KBr
Choline, V_{max}*	–	18.0 ± 1.0	18.0 ± 1.0
	+	14.0 ± 1.0	15.0 ± 1.0
Choline, Kt†	–	40.0 ± 2.0	40.0 ± 2.0
	+	42.0 ± 3.0	42.0 ± 1.0
D-myo-inositol, V_{max}*	–	11 ± 3	5 ± 1
	+	5 ± 1	4 ± 1
D-myo-inositol, Kt†	–	106 ± 1	108 ± 7
	+	103 ± 14	95 ± 17

HePC concentration was 10 μg/ml, mean and standard deviation of three independent duplicate experiments. * pmol/min/100 μg cellular protein. † μM.

(Table 4), and there were clear differences between the two cell lines. The KBr cells' uptake rate of HePC, after 2 h of incubation, was only 30% that of KB cells, but they were able to metabolise HePC at a 3-fold higher rate. However, it should be noted that, at 1 μg/ml HePC (close to the LC_{50} of KB), KB cells incorporated 1.7 nmol undigested HePC per million cells whereas KBr cells incorporated 5.7 nmol undigested HePC per million cells at 10 μg/ml HePC. At this HePC concentration, the KBr cells did not show any adverse effects regarding growth rate or loss of viability. On the contrary, to retain their HePC-resistance, KBr cells were routinely cultured in medium containing 10 μg/ml HePC. This seems to indicate that other mechanisms for the resistance of HePC are involved than slower uptake rates and faster metabolism of HePC.

DISCUSSION

Since the isolation of HePC-resistant cells by limiting dilution experiments or cloning in presence of HePC were

Table 4. Uptake and metabolism† of HePC*

	KB	KBr
Uptake after 1 h	2037 ± 1404	426 ± 240
Uptake after 2 h	3897 ± 1209	957 ± 367
Metabolism after 3 days:		
HePC‡	1675 ± 1031	5684 ± 3288
HePC in % of total radioactivity§	88	33

* Uptake was measured using radioactive HePC diluted to 10 μg/ml with cold HePC. Specific radioactivity after dilution was 2000 dpm/pmol. Uptake values are in pmol per 10^6 cells and are mean ± standard deviation from five independent experiments. † For metabolism studies, KB cells were incubated with 1 μg/ml HePC and KBr cells with 10 μg/ml HePC for a period of 3 days. In both cases, radiolabelled HePC was diluted with cold HePC to a specific radioactivity of 200 dpm/pmol. Given are values for unchanged HePC in pmol/10^6 cells, calculated as mean ± standard deviation from three independent experiments. ‡ Amount of non-metabolised HePC per 10^6 cells after 3 days of exposure to 1 μg (KB, LC_{50} 1.2 μg/ml) or 10 μg (KBr, LC_{50} 37 μg/ml) HePC per millilitre medium. Values are mean ± standard deviation of three independent duplicate experiments. § Total radioactivity was counted from a known number of cells in a Packard Tricarb liquid scintillation counter. The ratio of radiolabelled HePC to total radiolabel incorporated was determined using a Berthold linear analyser.

unsuccessful, a slow adaptation process to HePC at increasing concentrations was chosen. For this laborious procedure, a starting concentration of 0.01 μg/ml (23.5 nM) was selected. At this concentration, the cells would survive. Initially, minute increments of 0.01 μg/ml were made to the HePC concentration that were increased gradually to approximately 0.1 μg/ml. Yet, at approximately 0.4 μg/ml (0.94 μM, weeks 17–21), the cells showed a lower viability and a slower growth rate. However, by maintaining the HePC concentration at this level, we were able to adapt the cells slowly. After 4 weeks, the cells had recuperated and a first adaptation of the cells had taken place. At a concentration of 0.6 μg/ml (1.41 μM, weeks 24–28), a second crisis was observed, where the cells needed around 6 weeks to adapt to the HePC concentration. It is noteworthy that these two events of difficulties in adaptation were centered around the IC_{50} value of the original cells (0.5 μg/ml, 1, 2 μM), a concentration at which we were not able to obtain HePC-resistant cells in earlier experiments.

After the second period of difficulty, the concentration of HePC could be increased in larger increments (0.1 μg/ml), and the cells were rapidly brought to a concentration of 3.6 μg/ml (8.4 μM, weeks 48–51), a concentration already three times the LC_{50} of the parent cell line. At this stage the cells were almost 1 year in the adaptation process, which may explain the tolerance for this rather high concentration of HePC. At 3.6 μg/ml, however, a third adaptation threshold was observed, and again the cells had to be cultured at this level for a 4 week period. At this time, directly after the cells were again increasing their growth rate, an expansion of the culture was performed and cells were harvested and frozen as a back-up stock. A last hurdle was met at a concentration of 5.5 μg/ml (12.9 μM, weeks 55–59), after which event a second back-up stock was taken at 6 μg HePC/ml (14.1 μM). Subsequently, the cells were rapidly adapted and the process was stopped after a concentration of 10 μg/ml (23.5 μM) was reached at week 63. From the time curve of the adaptation in Figure 1, it is clear that the initial adaptation was the most time consuming. This seems to indicate that the cells are very sensitive to low concentrations of HePC, and that only after several adaptation stages, could the cells more easily tolerate higher concentrations of HePC. This is in accordance with the fact that it was not possible to select cells that were HePC-resistant when starting at higher concentrations. A similar failure to select HePC-resistant cells has been reported by Hilgard and associates [27], who came to the conclusion that their cells could not be made resistant to HePC. However, as shown here, a slow adaptation to increasing concentrations of HePC is possible.

The resistant cells maintained their level of HePC-tolerance for approximately 5 weeks. After this time, a rapid decrease in both IC_{50} and LC_{50} values was observed. After about 10 weeks, the cells reached a plateau, with IC_{50} and LC_{50} values approximately twice that of the original cells. This seems to indicate that the KBr and KB cells are not genetically different. Therefore, we believe that the two cell lines are a good model for better understanding the mechanism of action of HePC. Differences in protein expression could possibly provide information on which intracellular systems are involved in the mechanism of action of HePC.

Since the main biochemical parameters of both cells, e.g. protein, phospholipid and cholesterol content, were not greatly different, the differences between the cells must be more specific. The fact that uptake of HePC is slower in KBr cells than in KB cells, and metabolism of HePC is 3-fold faster in KBr cells than in KB cells, may partly explain a higher tolerance of KBr for HePC. However, at 1 μg/ml HePC, the KB cells showed severe damage at an internal concentration of HePC of 1.7 nmol per million cells, whereas at 10 μg/ml KBr cells were not affected but had an internal concentration of 5.7 nmol per million cells. This points to an induced tolerance for HePC based on mechanisms other than slow uptake or faster metabolism, more so since KBr cells were maintained routinely in 10 μg/ml HePC, whereas KB cells could not survive in 1 μg/ml HePC for more than 3 days.

Interestingly, KBr cells showed a 2-fold slower rate of uptake of inositol, similar to that of KB cells in the presence of HePC. This effect on inositol uptake seems specific and not due to general membrane effects of HePC, since there was no effect on the rate of uptake of choline for both KB and KBr cells. The amount of phosphatidylinositol, however, was similar in both cell types.

To our knowledge, this is the first report on the induction of resistance in KB cells to an ether-lysophospholipid analogue that was concluded successfully, although Rosen and associates reported the induction of tolerance to an analogue of HePC, OPP, after prolonged incubations with the compound [28]. The fact that the resistant KB cell is derived directly from a very sensitive cell line gives us the opportunity to investigate differences in the gene expression and translation process of proteins between the cells that are not due to cell origin, but are most probably related to the adaptation of the cells to HePC. These differences, therefore, will help us to find patterns that are involved in the mechanism of action of HePC and other ether-lipid analogues. Since the HePC-resistant cells are also resistant to the other ether-lipid analogues OMG-3PC and BM 41.440, we expect that all three ether-lipid analogues have a similar, if not identical, mode of action. The effect of resistance to HePC is not a general phenomenon, since the KBr cells did not show any change in

sensitivity to more commonly known chemotherapeutics such as doxorubicin or cytosine-arabinoside (data not shown). Future studies will focus on the protein composition of both cells and on changes in gene-expression.

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