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Effects of Hexadecylphosphocholine on Membrane Phospholipid Metabolism in Human Tumour Cells

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Hexadecylphosphocholine (HePC) is an analogue of the antiproliferative alkyllysophospholipds (ALP). As these lipid-like compounds interfere with membrane lipid metabolism at several sites, we studied the effects of HePC on uptake and metabolism of inositol and choline, two important phospholipid precursor molecules in two sensitive cell lines, Raji and KB, and in a resistant variant of KB cells, KBr. HePC substantially inhibited the membrane uptake of inositol and of choline in KB and Raji. Inositol uptake of KBr cells was constitutively low and was not further decreased by HePC. In all three cell lines, uptake inhibition of choline was less pronounced. Uptake inhibition showed characteristics of a non-specific effect, probably due to the physicochemical properties of HePC as a "lyso" structure. Decreased uptake of inositol did not affect phosphoinositide synthesis. Cellular phosphatidylcholine (PC) metabolism seemed to be affected through inhibition of choline incorporation and enhancement of PC degradation in the two sensitive cells. In KBr cells, these effects were not observed.

Key words: hexadecylphosphocholine, alkylphosphocholines, alkyllysophosphatidylcholines, etherlipids, phospholipid metabolism

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INTRODUCTION

ALKYLPHOSPHOCHOLINES (APC) are a new group of antiproliferative agents, derived from alkyllysophosphatidylcholines (ALP) following structure variation studies which examined their cytostatic and cytotoxic characteristics [1–5]. The main respresentative of this group is hexadecylphosphocholine (HePC). This compound showed remarkable selective antineoplastic properties in a variety of different tumours and tumour cell lines, with little or no toxic effects on normal cells [6, 7]. Although clinical investigations have already led to an application of HePC for the treatment of cutaneous metastasis of mammary carcinoma, little is known about the biochemical mechanisms by which HePC and probably also ALP mediate their biological effects. As APC and ALP are predominantly incorporated into cellular lipid compartments, it is conceivable that they interfere with membrane lipid composition or metabolism. Indeed, Herrmann and associates showed a reduced incorporation of oleic acid into phosphatidylcholine (PC), enhanced deacylation of PC and increased incorporation of fatty acids into triacylglycerols when different cell lines were exposed to the ALP prototype, ET-18-OCH_3 [8]. Vogler and associates reported a disturbance of PC synthesis in ET-18-OCH_3-treated HL60 cells caused by reduced choline incorporation and inhibition of lysophosphatidylcholine (LPC) reacylation [9]. Modolell and associates also described a decreased choline incorporation into PC and an enhanced accumulation of oleic and linoleic acid in the neutral lipid fraction [10].

Our intention was to investigate the effects of HePC on cellular phospholipid metabolism during two processes. Firstly, at the level of cellular uptake of choline and inositol, two important precursor molecules of phospholipid synthesis; and secondly, at the level of incorporation of these precursors into phospholipids. These investigations were performed on the human cell lines, Raji and KB, both susceptible to HePC, and on a HePC resistant variant of KB, KBr.

MATERIALS AND METHODS

Cell culture

The human Burkitt lymphoma cell line, Raji, and the epidermoid cancer cell line, KB, were obtained from the American Type Culture Collection, Rockville, U.S.A. Cells were grown in Click's/RPMI 1640 medium with 20 mM HEPES pH 7.3, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM glutamine

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and 10% FCS (fetal calf serum) at 37°C and 5% CO_2 (Gibco, U.K.). The HePC resistant variant of the KB cell line was established by continuous growth of culture with escalating HePC concentrations (from Asta Pharma AG Frankfurt/Main, Germany) over 12 months. The initial HePC concentration was 0.1 μg/ml. After 1 year, these KB cells tolerated HePC doses of 10 μg/ml without any signs of growth retardation or loss of viability as determined by cell count in a Neubaur chamber and trypan blue dye exclusion tests. The KBr line is permanently cultured in medium with HePC. Proliferation assays and visual examination show a homogeneous cell population. Further characterisation is still in progress.

Determination of phosphate, cholesterol and protein

Cell lipids were extracted according to the method of Bligh and Dyer [11]. Phospholipids were hydrolysed in 6 N sulphuric acid at 300°C for 1 h. The resulting inorganic phospate was analysed by the method of Eibl and Lands [12]. Cholesterol was measured using a commercially available kit (Sigma) by oxidation of cholesterol to cholestenone and hydrogen peroxide, and oxidation of dimethoxybenzidine in the presence of horseradish peroxidase in a second reaction. Final reaction products of cholesterol and phosphate were quantified photometrically in a Hitachi-spectrophotometer at 500 nm and 660 nm, respectively. Cellular protein content was determined according to Peterson's modification of the method of Lowry [13].

Thin layer chromatography

For separation and analysis of different phospholipids, the organic phases of Bligh/Dyer cell extracts were spotted on HPTLC silica gel 60 plates (Merck, Germany) with a semi-automated applicator (Linomat IV, Camag, Germany). For separation of phospholipids, a solvent system containing chloroform, methanol, triethylamine and water 30/34/35/8 (by vol.) or chloroform, methanol, acetic acid and water 60/50/2 (by vol.) was used (Baker, The Netherlands). Inositol-containing phospholipids were resolved on oxalate impregnated silica gel plates in chloroform, acetone, ethanol, acetic acid and water 80/30/26/24/14 (by vol.).

Cellular phospholipid labelling

5×10^5 to 2×10^6 cells/10 ml were cultured for 24 h in the presence of [^{14}C]choline (1 μCi/ml specific activity 55 mCi/mmol), [^{3}H]inositol (10 μCi/ml specific activity 20 Ci/mmol) or 1-palmitoyl-lyso-phosphatidyl[^{3}H]choline (1 μCi/ml) (Amersham, Germany). Organic phases of cell lipid extracts were separated by thin layer chromatography as described above. Distribution of radioactivity was measured in a Berthold Linear analyser (Tracemaster 20, Berthold, Germany). Lipids were then visualised and identified in comparison to standards by dipping the plates in copper sulphate solution and charring at 180°C or staining with Coomassie brillant blue.

Uptake of small molecules

In uptake kinetic experiments, $2–5 \times 10^6$ cells were incubated at 37°C in the presence of 47.5 μM (0.25 μCi/ml) [^{3}H]inositol, [^{14}C]choline, [^{14}C]methionine (specific activity 53 mCi/mmol) or [^{14}C]deoxyglucose (specific activity 56 mCi/mmol). Incubations were stopped by either sedimenting the cells rapidly through a bromododecane layer or by adding a 10-fold volume of ice-cold medium and washing the cells three times. Incorporated radioactivity was quantified in a Packard 1900 Ca β-counter by liquid scintillation counting.

Table 1. Proliferation of Raji, KB and KBr cells

Cell line	IC_{50} (μg/ml)	LC_{50} (μg/ml)
Raji	2.9 ± 1.7	24.0 ± 2.4
KB	0.6 ± 0.2	1.2 ± 0.9
KBr	21.4 ± 2.6	35.0 ± 2.9

IC_{50} and LC_{50} values were calculated from dose–response assays performed with escalating concentrations of HePC for 48 h. LC_{50} values represent the HePC concentrations at which 50% of the cells were killed measured by trypan blue dye uptake, IC_{50} values show HePC concentrations necessary to produce a half maximal decrease of cell count in comparison to untreated control cultures. Data are the mean values ±S.D. of four independent experiments.

RESULTS

After approximately 12 months of incubation with gradually increasing concentrations of HePC, a HePC-resistant variant of the KB cell line, KBr was established. Table 1 shows proliferation data of this variant compared with its parental line KB and Raji cells. KBr cells tolerated approximately 30-fold higher HePC concentrations than KB. There seem to be two different modes of susceptibility towards HePC. In Raji cells, HePC showed cytostatic effects at low concentrations of approximately 3 μg/ml whereas cytotoxic effects required much higher doses of approximately 24 μg/ml. In KB and KBr cells, the HePC doses for cytotoxic and cytostatic effects were in a narrow range. The reason for this phenomenon is currently unknown.

The analysis of cellular phospholipid and cholesterol content (Table 2) revealed almost identical amounts of both membrane lipid fractions in KB and KBr. Raji cells, being of lymphoid origin and of smaller dimensions, contained far less membrane lipids. Regardless of their size, all three cells show a very similar phospholipid to cholesterol ratio. Therefore, a direct correlation between phospholipid or cholesterol content or phospholipid to cholesterol ratio and cellular susceptibility towards HePC is not evident.

In order to investigate cellular phospholipid synthesis, cells were incubated with [^{14}C]choline or [^{3}H]inositol and treated with HePC. Incorporation of the radioactive label into phospholipids was analysed by thin layer chromatography after extraction of total cellular lipids (Table 3). In Raji and KB cells, a dose-dependent overall reduction of [^{14}C]choline incorporation due to a decrease of label incorporation in PC of approximatley 30% in Raji and 40% in KB cells was observed. In KBr cells HePC did not lead to any changes of [^{14}C]choline incorporation. In all three cell lines radioactive label distribution in lysophosphatidyl-

Table 2. Phospholipid and cholesterol content of Raji, KB and KBr cells

Cell line	Phospholipid (nmol/10^6 cells)	Cholesterol (nmol/10^6 cells)	P/C ratio
Raji	35.5 ± 2.0	8.7 ± 1.8	4.0
KB	78.2 ± 4.6	17.7 ± 1.2	4.4
KBr	77.1 ± 5.1	20.4 ± 2.3	3.7

Data represent mean values of three to six independent determinations ±S.D. P/C ratio, phospholipid/choline ratio.

Table 3. Incorporation of [^{14}C]choline and [^{3}H]inositol into cellular phospholipids of Raji, KB and KBr

Cell line	HePC (μg/ml)	[^{14}C]choline (%) LPC	SM	PC	[^{3}H]inositol (%) PIP_2	PIP	PI
Raji	0	0.6†	3.4*	96.0*	2.8*	4.9†	92.3†
	2	0.5	4.7	81.7	1.8	6.0	91.3
	4	0.6	4.5	61.8	5.4	4.6	88.4
KB	0	2.9*	6.7	91.0	5.1†	15.4	78.5†
	0.5	1.9	4.2	66.7	5.1	14.0	76.6
	1	1.8	5.5	52.8	5.6	11.1	81.5
KBr	0	3.3†	9.0†	87,7†	11.2†	10.4†	78.4†
	10	2.8	10.2	89.9	10.0	9.6	74.6

The accumulation of radioactivity in various phospholipids was quantified in a linear analyser and expressed as percentage of incorporation into total phospholipids. Untreated control cultures were set at 100%. Data are mean values of four different experiments. S.D. was in the range of 0.12–9.31%.
*$P < 0.05$; †$P > 0.05$.

choline (LPC) and sphingomyelin (SM) was unaffected. In Raji cells, [^{3}H]inositol incorporation showed a slight increase of the radioactive label in phosphatidylinositolbisphosphate (PIP_2) and a concomitant loss in phosphatidylinositol (PI) after HePC treatment. KBr cells *per se* contain higher amounts of radioactive label in PIP_2 compared to KB. HePC obviously has no effect on the radioactive label redistribution in these cells. An overall decrease of [^{3}H]inositol incorporation was also not apparent.

The pronounced decrease of [^{14}C]choline content in cellular PC in Raji and KB cells was also related to an enhanced degradation of PC (Table 4). When cellular PC was labelled with 1-palmitoyl-lyso-phosphatidyl[^{3}H]choline, substantial amounts of radioactive label were shifted from PC to SM in cells treated with HePC, probably through activation of a direct transfer of the phosphocholine group.

We also investigated the cellular uptake of choline and inositol

Table 4. Incorporaton of 1-palmitoyl-lyso-phosphatidyl [^{3}H]choline into cellular phospholipids of Raji and KB

Cell line	HePC (μg/ml)	1-palmitoyl-lyso-phosphatidyl[^{3}H]choline (%) LPC	SM	PC
Raji	0	2.5*	9.6*	87.9†
	2	4.3	17.2	76.9
	4	5.6	21.5	73.7
KB	0	1.8*	12.1*	86.1†
	0.5	3.4	17.3	78.4
	1	2.7	17.0	77.3

Radioactive label distribution was determined by a linear analyser and expressed as percentage of incorporation into total phospholipids. Untreated control cultures were set at 100%. Data are mean values of three independent experiments with S.D. being 0.11–9.10%.
*$P < 0.05$; †$P > 0.05$.

at the level of their specific membrane carrier systems. Figure 1 shows uptake experiments with [^{14}C]choline and [^{3}H]inositol in KB and KBr cells. For both substances, cellular uptake is saturable at higher concentrations. HePC treatment in both cell variants produced a small decrease of choline uptake of approximately 10%. In KB cells, inositol uptake was 2-fold higher than in KBr. In the latter cells addition of HePC did not further affect inositol uptake, whereas in KB cells inositol uptake was reduced by 50%. Uptake inhibition by HePC in Raji cells was very similar with 50% for inositol and 15% for choline (data not shown).

Calculations of V_{max} and K_T values by Lineweaver–Burk plot analysis in Table 5 reveal a mixed type of uptake inhibition for choline with a slightly decreased V_{max} and increased K_T. Inositol uptake inhibition in KB cells is clearly non-competitive. HePC incubation reduced V_{max} values by more than 50%. In KBr, V_{max} values were constitutively 50% lower than in KB, and HePC treatment did not produce a further decrease. Obviously, long-term exposure of KB cells to HePC, as in KBr, resulted in a very similar uptake inhibition of inositol as short-term incubation of 2 h with the same HePC concentration.

As both inositol and choline uptake were inhibited, it was interesting to see whether other nutrients showed reduced cellular incorporation. Figure 2 shows the uptake of [^{14}C]choline, [^{3}H]inositol, [^{14}C]methionine and [^{14}C]desoxyglucose in Raji cells treated with 10 μg/ml HePC for 2 and 24 h, respectively. HePC inhibited the uptake of all four substances in a time-dependent manner. Inositol uptake was the most affected.

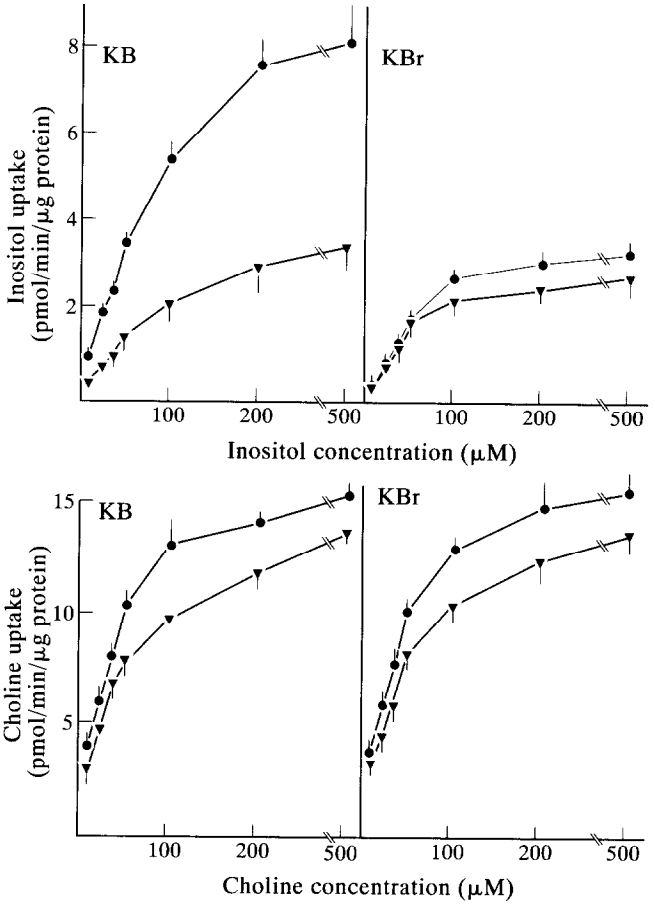

Figure 1. Uptake of [^{14}C]choline and [^{3}H]inositol in KB and KBr cells. Results are presented as mean values ±S.D. of three different experiments.

Table 5. Maximal transport velocity (V_{max}) and affinity (K_T) of carrier systems for inositol and choline

Cell line	HePC (μg/ml)	V_{max} (pmol/min/100 μg protein)	K_T (μM)
Choline			
KB	0	18	40
	10	14	42
KBr	0	18	40
	10	15	43
Inositol			
KB	0	11	106
	10	5	103
KBr	0	5	108
	10	4	97

Data were determined by Lineweaver–Burk plot analysis of [^{14}C]choline and [^{3}H]inositol uptake experiments shown in Figure 1.

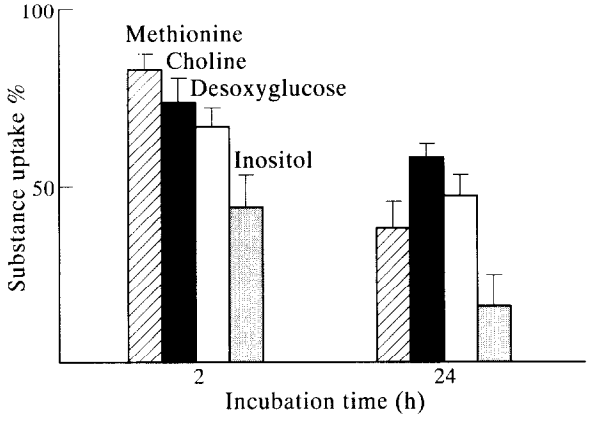

Figure 2. Uptake of [^{14}C]methionine, [^{14}C]choline, [^{14}C]desoxyglucose and [^{3}H]inositol in Raji cells. Data are expressed as percentage of uptake of untreated controls and represent mean values ±S.D. of three independent experiments.

However, other substances, such as the HePC analogue hexadecylphosphocho-*N*,*N*,*N*-trimethylhexanolamine ($HePC_6$) and 1-palmitoyl-sn-glycero-3-phosphocholine (ES-16-OH), two compounds with only weak or no antiproliferative activity, also produced a 30–60% inhibition of [^{3}H]inositol uptake in Raji cells similar to HePC at equimolar concentrations (Figure 3).

DISCUSSION

By stepwise exposure of cells to escalating doses of HePC, it was possible to adapt the KB cell line to high HePC concentrations. Analysis of this HePC resistant variant showed equal phospholipid and cholesterol content as well as the same phospholipid to cholesterol ratio as the original KB line. The phospholipid to cholesterol ratio was also comparable to Raji cells, which contain far less of these membrane lipids due to their smaller size. From these data, it is evident that differences in phospholipid to cholesterol ratio do not correlate with growth inhibitory effects of HePC. This is in contrast to findings of Diomede and associates [14] who showed that higher amounts of cholesterol when introduced to HL60, rendered these cells more resistant to ET-18-OCH_3. This effect may, of course, be specific for leukaemic cells.

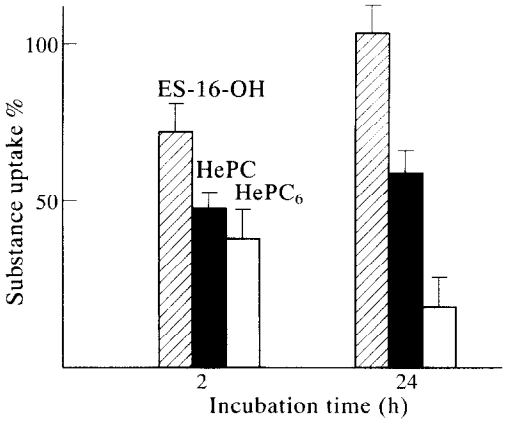

Figure 3. Effects of ES-16-OH, $HePC_6$ and HePC on [^{3}H]inositol uptake in Raji cells. Data are expressed as percentage of uptake of untreated controls and represent mean values ±S.D. of three different experiments.

HePC appears to inhibit the uptake of a variety of different cellular nutrients. In our opinion, this observation supports the idea of a non-specific membrane effect by HePC rather than a specifically targeted action at special carrier proteins. HePC and ALP, like ET-18-OCH_3, probably interfere with cellular uptake mechanisms by a general disturbance of membrane structures or lipid–protein interactions through the physical characteristics of these compounds as lysophospholipid structures, leading to a change of molecular conformation and activity of many carrier systems. This view is supported by several lines of evidence: firstly, uptake experiments with the physiological membrane lysophospholipid ES-16-0H and a HePC analogue $HePC_6$, a compound with identical physicochemical but only weak antiproliferative properties, show a similar uptake inhibition of inositol. The inhibitory effect of ES-16-OH is abrogated after a longer incubation period, most probably by reacylation of this molecule to PC. Secondly, carrier saturation studies and Lineweaver–Burk plot analysis reveal non-competitive inositol uptake inhibition. Hoffman and associates described very similar effects for ET-18-OCH_3 in HL60 cells [15] where not only was the uptake of choline and inositol affected, but so was that of methionine, desoxyglucose, histidine and arachidonic acid. These authors also noticed uptake inhibition of these nutrients when the cells were treated with LPC. Thirdly, the inhibitory action of HePC on inositol uptake is established very quickly. After 10 min exposure to HePC, inositol uptake is already reduced by 20%. At this time point, there is not enough HePC incorporated into the cells to produce typical HePC-induced biological effects [16, 17]. Fourthly, KBr cells maintained without HePC for several hours regain almost the same inositol uptake capacity as KB cells (data not shown).

A downregulation of membrane carrier molecules or other membrane proteins, as described for the epidermal growth factor receptor [18, 19], may also play a role. HePC may also induce uptake inhibition indirectly by affecting the Na/K-ATPase activity [20, 21] and thereby reducing the Na influx, a process coupled to several uptake mechanisms for different molecules. Interestingly, KBr cells, maintained permanently in HePC-containing medium, do not show any retardation in proliferation although they should contain less intracellular inositol as their inositol uptake is only 50% of normal KB cells. Perhaps KBr cells compensate for lower uptake by higher endogenous *de novo*

synthesis of inositol, or perhaps these cells do not need high intracellular inositol storages, as has been described for DMSO-differenitated HL60 cells [22].

However dramatic uptake inhibition of inositol may seem, for inositol-containing phospholipid synthesis and metabolism, it is obviously of no importance. In all three cell lines tested here, there was no impairment of PI, PIP or PIP_2 synthesis. In Raji cells a discrete accumulation of PIP_2 was observed with HePC treatment. This may reflect an inhibitory effect of this compound on a PIP_2-degrading enzyme, a PI-specific phospholipase C (PI-PLC), as reported earlier by Überall and associates in NIH 3T3 cells [23], and by Seewald and associates and Powis and associates for ET-18-OCH_3 in Swiss 3T3 and BG 1 cells [24, 25]. In contrast to inositol, [^{14}C]choline incorporation into PC was reduced by 30% in Raji and almost 40% in KB cells. This substantial effect is hardly the result of non-specific choline uptake inhibition, but could be mediated by an inhibition of CTP (phosphocholine-cytidylyltransferase) the rate limiting enzyme in PC synthesis via CDP-choline [26], as postulated by Geilen and colleagues [27], Haase and associates [28] and Wieder and associates [29]. Our own experiments, with direct labelling of PC by 1-palmitoyl-lyso-phosphatidyl[^{3}H]choline, prove that this synthesis block is paralleled by an enhanced degradation of PC, probably by transfer of a phosphocholine group to ceramide to form sphingomyelin as described by Voelker and Kennedy, and Fleer and colleagues [30, 31]. In this particular case, HePC seems to activate an enzyme with PC-specific phospholipase C (PC-PLC) characteristics, which hydrolyses PC to diacylglycerol and phosphocholine, or it enhances a direct phosphocholine exchange via a "phosphocholine-transferase". In the three cell lines we employed for our investigations, we did not observe an inhibition of LPC reacylation when cells were labelled with 1-palmitoyl-lyso-phosphatidyl[^{3}H]choline. This is in contrast to findings of Vogler and colleagues who described reduced activity of acyltransferase in ET-18-OCH_3-treated HL60 cells [9].

Although HePC susceptible cells, such as KB or Raji, can be discriminated from resistant cells, such as KBr, by a reduced PC synthesis with HePC treatment, it seems too early to postulate that the interference with PC metabolism is the mechanism by which APC or ALP lead to growth inhibition and cell death. Preliminary data from our laboratory show only insignificant changes of PC levels after HePC treatment for 24 h (data not shown). Haase and associates reported a pronounced decrease of choline incorporation in MDCK cells, of approximately 50% of control after only 2 h of HePC treatment. This was followed by only a slight reduction (17%) of PC content after 24 h of incubation [28]. This relatively long period required to produce a significant loss of PC does not correlate with the short HePC exposure time necessary to initiate typical biological effects of HePC, such as induction of differentiation and growth inhibition [17].

Taken together, these data indicate that HePC produces a non-specific disturbance of cellular uptake of several molecules which does not lead to a significant change of membrane phospholipid synthesis. At later stages of PC synthesis, HePC inhibits choline incorporation into PC and enhances PC degradation in HePC-susceptible cell lines, whereas in a resistant cell variant, the PC metabolism remains unchanged. There is, however, strong evidence arguing against PC metabolism as the main target for HePC-induced anitproliferative effects. HePC treatment leads to distinct changes in the membrane phospholipid pattern so that investigations in the near future should focus on possible interaction of HePC with special phospholipid subclasses and phospholipid-degrading enzymes (phospholipases) involved in second-messenger cascades. Here, subtle changes of certain phospholipid pools, not measurable in overall phospholipid metabolism studies, may have significant effects on the biological response of cells.

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