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Distribution of hexadecylphosphocholine and octadecyl-methyl-glycero-3-phosphocholine in rat tissues during steady-state treatment

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Summary. The distribution of the alkylphosphocholine hexadecylphosphocholine (He-PC) and the (alkyl)lysophospholipid 1-0-octadecyl-2-0-methyl-rac-glycero-3-phosphocholine (ET_{18}-OCH_3) was analyzed in rats. The compounds were given orally at a daily dose of 75 μmol/kg body weight. After 6, 11, and 18 days, three rats in each treatment group were killed and the drug concentration in various tissues and fluids was determined. With the exception of the kidney (He-PC) and brain (He-PC and ET_{18}-OCH_3), steady-state levels of the drugs could be achieved in all organs investigated and in serum. Maximal concentrations of He-PC were found in the kidney, adrenal glands, and spleen, whereas the highest concentrations of ET_{18}-OCH_3 were detected in the adrenal glands, spleen, and small intestine. The concentrations of He-PC exceeded those of ET_{18}-OCH_3 in most tissues by a factor of about 2–25. Since samples of urine and feces did not contain detectable amounts of the compounds, the absorption of both lipid analogues was assumed to be complete. The total amount of He-PC recovered after 6, 11, and 18 days was 15%, 12%, and 6%, respectively, and that of ET_{18}-OCH_3 was 1.3%, 0.8%, and 0.3%, respectively. This indicates that the bioavailability of He-PC and ET_{18}-OCH_3 is not controlled by differences in the uptake of the two drugs, but by differences in their metabolism. The results could explain the differing efficacy of these two compounds in their antitumor action in animal models.

Introduction

The alkylphosphocholine hexadecylphosphocholine (He-PC) and the (alkyl)lysophospholipid 1-0-octadecyl-2-0-methyl-rac-glycero-3-phosphocholine analogue (ET_{18}-OCH_3) can induce a great diversity of cellular events. Besides activation of macrophages to cytotoxic effector cells [1], induction of malignant cell differentiation [14, 15, 18], and inhibition of tumor-cell invasion in normal tissues [24, 27], these compounds show antitumor activity against tumor cells in vitro and in vivo [9, 14, 18, 21]. In contrast to the generally comparable cytotoxic and cytostatic effects of both compounds in vitro, remarkable differences in their antitumor effects in vivo have been reported [2, 21]. In vivo, He-PC exerts higher antitumor efficacy as compared with equimolar doses of ET_{18}-OCH_3 [3]. Although initial pharmacokinetics studies of He-PC in mice have been reported [4], little is known in the case of ET_{18}-OCH_3.

To investigate the tissue distribution of ET_{18}-OCH_3 in comparison with that of He-PC in rats after oral administration, we used a sensitive and reproducible method described by Rustenbeck and Lenzen [23] employing thin-layer chromatography combined with densitometry. This procedure results in the sensitive detection of ET_{18}-OCH_3 and He-PC to a lower limit of about 1 nmol/g tissue. The results obtained demonstrate strong differences in the tissue and serum concentrations of the two compounds that might explain the different in vivo susceptibility of experimental tumors.

Materials and methods

Lipids. He-PC and ET_{18}-OCH_3 were synthesized in our laboratory as described elsewhere [5–7]. The chemical structures of the two compounds are given in Fig. 1.

Animal experiments. Female Wistar rats (Zentralinstitut für Versuchstierkunde, Hannover, FRG) weighing 180–200 g were kept under conventional, controlled conditions. The lipids were dissolved in distilled water and diluted to the desired concentration. Both compounds were given at a dose of 75 μmol/kg in a 0.2-ml solution via a stomach tube. The rats were treated daily (five times a week) for up to 3 weeks. After the indicated intervals, the animals were killed and blood samples were taken by cardiac puncture. After exsanguination of the animals by infusion of 0.9% NaCl into the left ventricle, the organs were removed, weighed, and kept frozen at –80° C until their use. At 24 h after start of treatment, feces were removed from the cages and analyzed for the content of the test compounds. During the whole study period, urine samples were taken and analyzed for the test compounds.

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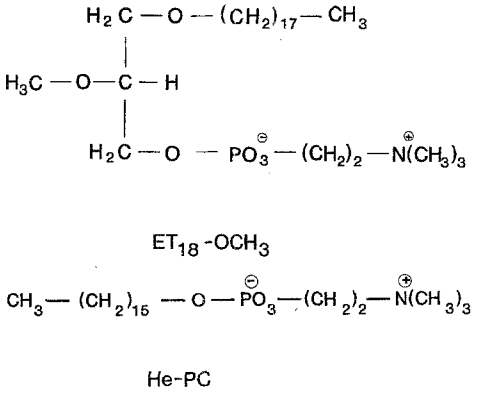

Fig. 1. Chemical structures of 1-0-octadecyl-2-0-methyl-rac-glycero-3-phosphocholine (*ET18-OCH3*) and hexadecylphosphocholine (*He-PC*)

Lipid extraction. In all, 0.1–0.5 g of each organ was cut into small slices. After the addition of 3 ml $CHCl_3$:MeOH:10% NaCl (2:2:1, by vol.) the mixture was homogenized at 3,000 rpm in an ice-water bath by the application of 10 strokes in an Elvehjem-Potter with a Teflon pestle driven by a Homogen homogenizer from Schütt (Göttingen, FRG). Sticky material from the pestle and the homogenizer was washed from the other material with 2 ml water. For lipid extraction, the tissue homogenates and serum samples (0.2–0.5 ml) were mixed with 2 ml $CHCl_3$:MeOH (2:1, v/v) and vortexed for 30 s. For phase separation, the tubes were spun at 3,000 *g* for 5 min in a centrifuge from Sigma Christ (Osterode, FRG). The proteins formed a precipitate at the interface. The lower chloroform layer contained the lipids and was transferred with a Pasteur pipette to a second set of test tubes. Extraction of the upper protein/water layer was repeated twice with 2 ml $CHCl_3$:MeOH (3:1, v/v) and 2 ml $CHCl_3$. The chloroform extracts were combined and the solvent was removed carefully under a stream of nitrogen. Depending on the ether lipid concentration expected in the samples, the dried extracts were dissolved in a volume of 0.1–1 ml $CHCl_3$:MeOH:water (30:60:8, by vol.).

High-performance thin-layer chromatography. For high-performance thin-layer chromatography, HPTLC plates were prerun with a mobile phase, $CHCl_3$:MeOH:trimethylamine:water (30:35:34:8, by vol.). Samples and standards were automatically streaked onto the HPTLC plates with a Linomat IV apparatus (Camag, Berlin, FRG). The sample volume was set at between 2 and 20 µl. The samples were applied as 5-mm lines located about 10 mm from the lower edge of the plate; the distance to the next sample was 4 mm. The plates were developed in glass tanks with the mobile phase to a distance of about 1 cm from the upper edge, which resulted in a total distance of 7.5 cm. After the plates had been dried at 180° C for 10 min on a heating plate from Desaga (Heidelberg, FRG), the lipids were visualized by staining with cupric sulfate solution (10%, w/v) in phosphoric acid (8%, w/v) [28]. The HPTLC plates were dipped for 15 s in the dye solution and dried on the heating plate by increasing the temperature from 110° to 180° C within 7 min. The He-PC and ET_{18}-OCH_3 spots on the cooled plates were then read quantitatively using a CD 60 TLC scanner from Desaga at 530 mm.

In typical calibration curves for He-PC and ET_{18}-OCH_3, the absorbance increased linearly with increasing amounts of both compounds in the range between 0.1 and 1.5 nmol. The regression coefficient on different days was 0.995 ± 0.004 for both lipids. The deviations in the results calibrated from the theoretical values were less than 5%. After the extraction and estimation of He-PC and ET_{18}-OCH_3, their overall recovery from tissues and from serum ranged between 90% and 110%. The lower limit of detection for He-PC and ET_{18}-OCH_3 using the described method was 50 pmol.

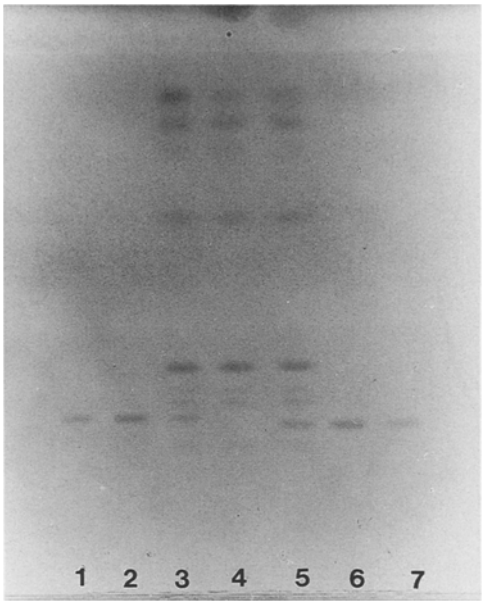

Fig. 2. Separation of He-PC and ET_{18}-OCH_3 from serum lipids by HPTLC. He-PC and ET_{18}-OCH_3 are located between lysophosphatidylcholine and sphingomyeline. Standard runs (*lanes 1, 2, 6, and 7*) indicate 0.5 (*lane 1*) and 1.0 nmol (*lane 2*) ET_{18}-OCH_3 and 1.0 (*lane 6*) and 0.5 nmol (*lane 7*) He-PC, respectively. *Lanes 3 and 5* correspond to serum samples containing 0.5 nmol ET_{18}-OCH_3 and He-PC, respectively. *Lane 4* represents a control serum

Results

The concentrations of the alkylphosphocholine He-PC and the lysophospholipid analogue ET_{18}-OCH_3 were measured in serum and various organs of rats after daily oral administration for 6, 11, and 18 days. Both compounds were separated from total lipid extracts of serum and tissues by HPTLC and were quantified by densitometric evaluation. This procedure resulted in the successful separation of the lysophospholipid analogues from other phospholipids in the samples. The lower detection limit was about 1 nmol/g tissue. A characteristic HPTLC run of serum samples is shown in Fig. 2. He-PC and ET_{18}-OCH_3 were clearly separated from lysophosphatidylcholine and sphingomyeline.

Figure 3 shows the distribution of both compounds in 13 organs and in serum of rats after 6, 11, and 18 days of drug administration. Each column represents the mean tissue concentration expressed in nanomoles per gram wet weight as determined in three animals. For He-PC, tissue levels of up to 100 nmol/g were measured in the brain, muscle, heart, and large intestine. Concentrations of between 100 and 500 nmol/g were found in the stomach, liver, skin, fat tissue, and serum. The highest concentrations were measured in the kidney, adrenal gland, lung, spleen, and small intestine. Tissue steady-state levels were obtained within 11 days. However, the brain and kidney showed an increase in drug concentration even after 11 days, although the brain concentration remained very low after 18 days (61 nmol/g) as compared with the concentration in the kidney (1,390 nmol/g).

In general, tissue concentrations of ET_{18}-OCH_3 were approximately 2–25 times lower than those of He-PC. As

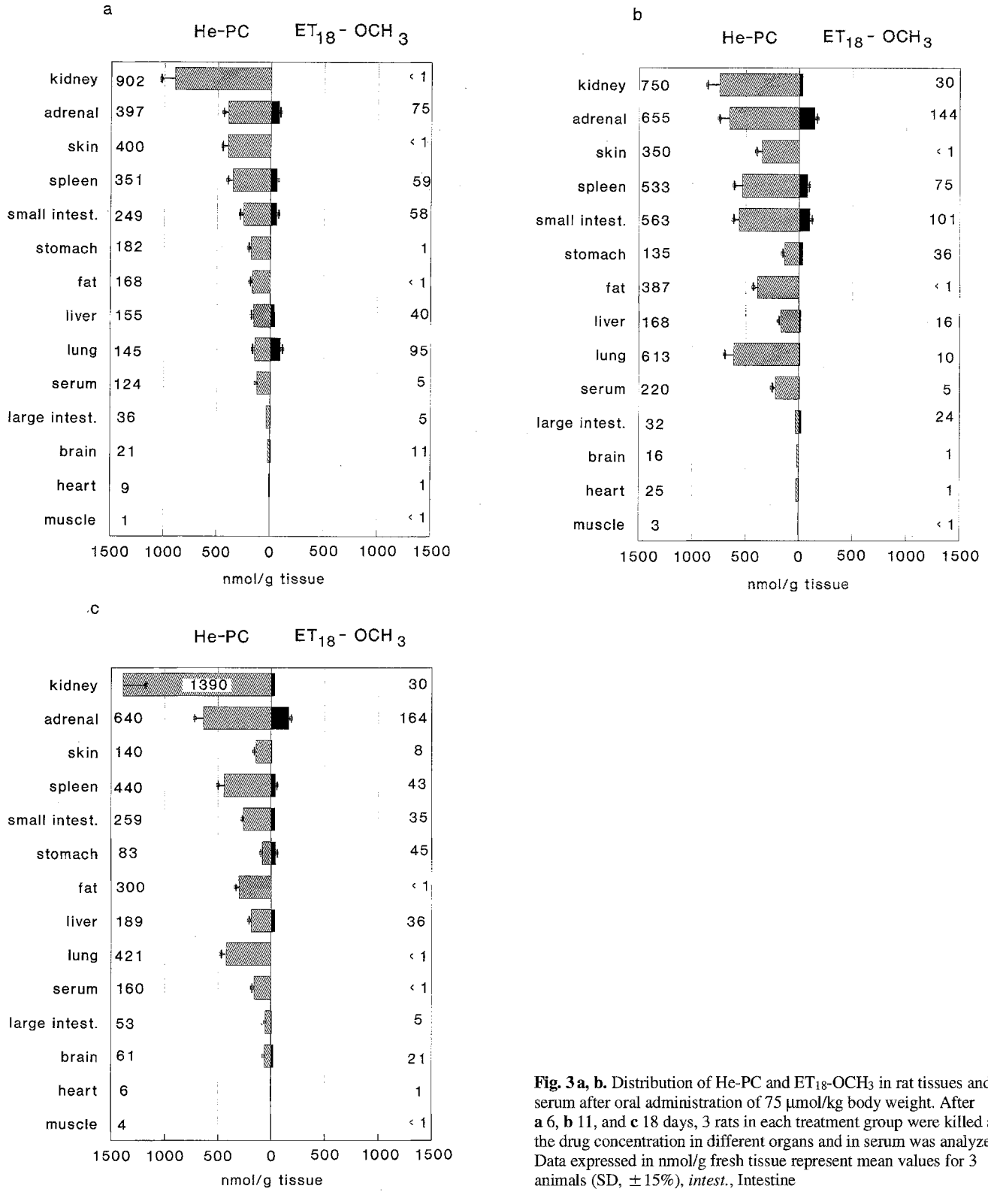

Fig. 3 a, b. Distribution of He-PC and ET_{18}-OCH_3 in rat tissues and serum after oral administration of 75 µmol/kg body weight. After **a** 6, **b** 11, and **c** 18 days, 3 rats in each treatment group were killed and the drug concentration in different organs and in serum was analyzed. Data expressed in nmol/g fresh tissue represent mean values for 3 animals (SD, ±15%), *intest.*, Intestine

indicated in Fig. 3, up to 24 nmol/g was found in the brain, heart, skin, large intestine, and serum, whereas in muscle and fat tissue the concentration of ET_{18}-OCH_3 was below the detection limit. Except for the adrenal gland, all investigated tissues revealed drug levels below 100 nmol/g. Comparable with He-PC, the concentration of ET_{18}-OCH_3 in the brain increased with time and did not show saturation within 3 weeks. Interestingly and in strong contrast to the findings for He-PC, the kidney concentrations of ET_{18}-OCH_3 were rather low and did not exceed the level of 30 nmol/g.

Discussion

The present study demonstrates that after oral application to rats, the lysophospholipid analogues He-PC and ET_{18}-OCH_3 are distributed throughout the body. Most interestingly, the tissue concentrations of He-PC in most of the organs tested exceeded those of ET_{18}-OCH_3 by a factor of about 2–25. These strong differences in organ levels and body fluids might be explained by differences in either the absorption or the metabolism of these compounds.

Recently, the bioavailability of He-PC was studied after oral and i.v. administration in mice. For tritium-labeled He-PC, complete absorption of the compound was reported [4]. Unfortunately, this study was based on the measurement of radioactivity; the structural integrity of He-PC in the different tissues was not verified. In the present study, we found that feces obtained from rats at 24 h after oral application did not contain detectable amounts of He-PC or ET_{18}-OCH_3. This observation may indicate the complete absorption of both compounds from the gut; however, it could also indicate their extensive metabolism either in the gut or elsewhere.

The cumulative rates of recovery of He-PC from the different organs tested and from serum after 6, 11, and 18 days were 15%, 12%, and 6%, respectively. In the case of ET_{18}-OCH_3, these values were lower, reaching only 1.3%, 0.8%, and 0.3%, respectively. Since elimination of He-PC and ET_{18}-OCH_3 apparently could not be detected in feces and urine, their rapid metabolism may explain the low rates of recovery of the compounds from the different tissues.

In leukemia cell lines in vitro, He-PC labeled in the choline-methyl group is slowly degraded. A major pathway of degradation was suggested to involve a transfer of the phosphocholine group to diacylglycerol, yielding (diacyl)phosphatidylcholine and hexadecanol [8]. This reaction was found earlier for ET_{18}-OCH_3 labeled in the polar head group, confirming the data obtained by other groups using ET_{18}-OCH_3 labeled in the octadecyl chain of the molecule, whereby 1-0-octadecyl-2-0-methyl glycerol was found, as expected from the above-described transfer reaction [26]. This pathway was likewise reported to take place in vivo, since besides choline and phosphocholine, the time-dependent formation of 1,2-diacylphosphatidylcholine in mouse liver after i.v. administration of hexadecylphospho(methyl-^{3}H)-choline could be observed [4]. From the biodistribution data and the strong difference in the tissue and serum concentrations of He-PC and ET_{18}-OCH_3, one would expect a faster degradation of ET_{18}-OCH_3. In fact, a half-life of approximately 36 h was reported for the radiolabeled compound [12]. This value is close to the half-life of 27 h reported for the structurally related thioether analogue 1-hexadecylmercapto-2-methoxymethyl-rac-glycero-3-phosphocholine [13]. In contrast, the half-life of He-PC after the administration of a single dose to rats has been shown to be 96 h [29].

Most importantly, the marked differences in tissue and serum concentrations may explain, at least in part, the differences in the antitumor effect of the two compounds. For instance, in chemically induced rat mammary carcinomas, the antitumor activity of ET_{18}-OCH_3 is merely cytostatic [2], whereas He-PC exhibits cytocidal effects, resulting in complete regression of the tumors [21]. In a study on the antitumor efficacy of He-PC and ET_{18}-OCH_3 in methylnitrosourea (MNU)-induced rat mammary carcinomas, both drugs were given orally at 51 μmol/kg for 6 weeks [3]. The efficacy of the drugs was compared on the basis of differences in tumor volumes expressed as a percentage of untreated control values (T/C%). According to the criteria of the National Cancer Institute, a significant inhibition of tumor growth is achieved if this value is below 43% [30]. Whereas ET_{18}-OCH_3 did not reach this value (59%), He-PC produced a value of 13% and some animals showed complete tumor remission. Moreover, a clear-cut dose-activity relationship could also be demonstrated for He-PC [3].

Despite their differing pharmacokinetic properties, it seems that there are further substantial differences between these two compounds. ET_{18}-OCH_3 was repeatedly shown to exhibit immunomodulatory activity [1], whereas He-PC showed no such activity in appropriate test systems [16]. Furthermore, the pronounced selectivity of He-PC for certain experimental tumors is outstanding. Whereas ET_{18}-OCH_3 showed some activity in transplantable rodent tumors such as P388 leukemia, Lewis lung carcinoma, and L1210 leukemia, the action of He-PC was confined to models such as autochthonous rat tumors and certain xenografts such as the KB-tumor [17].

Several modes of action have been postulated to account for the antineoplastic effect of synthetic phospholipid analogues. Enhancement of the cytotoxic properties of macrophages [20] and alteration of phospholipid metabolism have been described [11, 19]. More recently, inhibition of protein kinase C [10, 22, 31] and phospholipase C [25] as well as inhibition of Na^+- and K^+-ATPases [31] have been reported. However, it remains to be established whether one of these events ultimately leads to cessation of the proliferation of cancer cells. Possibly, the antineoplastic effect may result from a summation of several cellular events mediated by synthetic phospholipid analogues.

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