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Cytotoxicity and Metabolism of Alkyl Phospholipid Analogues in Neoplastic Cells[1]

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ABSTRACT

The cytotoxic response of several types of neoplastic cells to analogues of unnatural alkyl phospholipids (*e.g.*, *rac*-1-hexadecyl-2-methoxy-glycero-3-phosphocholine) has been partially attributed to their accumulation as a result of the low activity of the alkyl cleavage enzyme (a tetrahydropteridine-dependent monooxygenase) in tumor cells. We tested this possibility by comparing the alkyl cleavage enzyme activity in cells that exhibit differences in sensitivity toward the cytotoxic effects of the *rac*-1-hexadecyl-2-methoxy-glycero-3-phosphocholine. Human promyelocytic leukemia cells (HL-60), a cell line highly sensitive to the cytotoxic alkyl phospholipid analogue, possessed an alkyl cleavage enzyme activity (0.25 pmol/min/μg protein) similar to that found in three cell types known to be relatively resistant to the cytotoxic activity of the analogue: immature human promyeloblastic leukemia cells (K562) (0.22 pmol/min/μg protein), human polymorphonuclear neutrophils (0.34 pmol/min/μg protein), and Madin-Darby canine kidney cells (0.37 pmol/min/μg protein). Moreover, our results indicate that the cytotoxic *rac*-1-octadecyl-2-methoxy-glycero-3-phosphocholine analogue is not a substrate for the alkyl cleavage enzyme with an active microsomal preparation of the enzyme from rat liver; cleavage of this analogue was 200-fold less than the rate obtained with 1-octadecylglycerol as substrate. In cultures of either sensitive or resistant type cells, approximately 90% of the added *rac*-1-[9′,10′-^{3}H]octadecyl-2-methoxy-glycero-3-phosphocholine was not metabolized during a 24-h incubation. The amount of radiolabel in fatty acids, a major product of alkyl cleavage activity, was small, and essentially identical amounts were produced in all four cell types [3.1 ± 0.2% (SD)]. These data indicate that differences in the cellular activities of the alkyl cleavage enzyme are not responsible for the differential cytotoxic responses between normal and specific types of neoplastic cells toward *rac*-1-octadecyl-2-methoxy-glycero-3-phosphocholine. On the other hand, the cellular uptake of the alkyl phospholipids could be a factor in explaining the cytotoxic response of certain tumor cells, since more radiolabeled 1-octadecyl-2-methoxy-glycero-3-phosphocholine was associated with the susceptible HL-60 cells than with the resistant cell types.

Autoradiography revealed that the radiolabeled 2-methoxy analogue accumulates at the periphery of HL-60 leukemia cells, whereas the label was more uniformly distributed in polymorphonuclear neutrophils and K562 cells. In contrast, the relatively nontoxic naturally occurring 1-[1′,2′-^{3}H]alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor), an important cellular mediator involved in hypersensitivity and cardiovascular responses, is rapidly metabolized by HL-60 cells and appears evenly distributed throughout the leukemia cells as judged by autoradiography. Data from these experiments indicate that the unnatural alkyl phospholipids accumulate at the surface membrane of "sensitive" cancer cells, the site where their cytotoxic action appears to be elicited.

INTRODUCTION

Certain unnatural alkyl phospholipids have been shown to be highly cytotoxic to certain neoplastic cells compared to normal cells (1, 2). The chemical structure of this type of antineoplastic phospholipid is closely related to that of a recently identified group of novel bioactive phospholipids (3–5) known as PAF[4] (1-alkyl-2-acetyl-GPC). In contrast to the antineoplastic phospholipids, PAF possesses a different type of profound biological activity that under specified conditions can cause the aggregation and degranulation of platelets and neutrophils as well as induce hypotensive, inflammatory, and anaphylactic responses (6, 7).

Two unnatural alkyl phospholipid analogues that have elicited the most potent selective cytotoxic responses from tumor cells are 1-octadecyl-2-acetamide-GPC and 1-hexadecyl-2-methoxy-GPC (8, 9). Native PAF, on the other hand, is relatively innocuous in terms of cellular toxicity (8). Structural features required for the selective antineoplastic activity of these unnatural phospholipids are: (*a*) an ether-linked alkyl moiety at the 1-position; (*b*) an apparent nonmetabolizable group at the 2-position; and (*c*) a quaternary phosphobase at the 3-position of the *sn*-glycerol moiety (9–12).

One explanation proposed (10) as a basis for the difference in the cytotoxic activities of the alkyl phospholipid analogues between normal cells and tumors is that they accumulate in the neoplastic cells due to the diminished capacity (13) of such cells to hydrolyze the substituents at the *sn*-1 and *sn*-2 positions. Presumably normal cells might cleave the alkyl moiety of the PAF analogues (10, 14, 15) via the Pte·H_4-dependent alkyl cleavage enzyme that has been well characterized in rat liver tissue (13, 16–20). However, the substrate specificity of this enzyme would require that the methoxy or acetamide groups at the *sn*-2 position be metabolically removed first since alkyl cleavage occurs only when the *sn*-2-position of glycerophospholipids is unsubstituted (16, 19).

Several investigations have supported the role of the alkyl cleavage enzyme in explaining the antineoplastic action of the unnatural alkyl phospholipid analogues; for example, Andreesen *et al.* have reported that the metabolism of the 1-octadecyl-2-methoxy-GPC analogue by alveolar macrophages (10) and human bone marrow cells (14) is greater than by human leukemic cells; also, Berdel *et al.* (15) found the lowest alkyl cleavage enzyme activity in rat brain tumor cells that were most sensitive to the cytotoxic alkyl phospholipid analogues. However, despite the apparent relationship between the low alkyl cleavage enzyme activity and the antineoplastic properties of the 1-hexadecyl-2-methoxy analogue, there has never been any direct evidence presented as to whether the Pte·H_4-dependent alkyl cleavage enzyme can indeed utilize 1-alkyl-2-methoxy-GPC as a substrate.

In the present investigation we have attempted to clarify the role of the alkyl cleavage enzyme in the differential cytotoxic responses observed between certain neoplastic and normal cells after their exposure to the unnatural PAF analogues. The activity of the microsomal Pte·H_4-dependent alkyl monooxygenase from rat liver with *rac*-1-[9′,10′-^{3}H]octadecyl-2-methoxy-GPC as a substrate was determined; in addition, the metabolism of the labeled 2-methoxy analogue was measured in

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[4] The abbreviations used are: PAF, platelet-activating factor; Pte·H_4, tetrahydropteridine; HL-60, human promyelocytic leukemia cells; K562, human promyeloblastic leukemia cells; PMN, human polymorphonuclear neutrophil; MDCK, Madin-Darby canine kidney cell; GPC, *sn*-glycero-3-phosphocholine (*sn* unless otherwise specified).

cultured HL-60 cells [highly sensitive to the unnatural alkyl phospholipids (8, 11, 21)] and in cells [human K562 (21), MDCK, and human PMN (8)] known to be relatively resistant to the cytotoxic analogues. Autoradiographic techniques were also utilized to evaluate the location of the labeled 2-methoxy analogue of PAF in intact cells that had previously been shown to exhibit different cytotoxic responses toward the phospholipid analogues.

MATERIALS AND METHODS

Chemicals. *rac*-1-Octadec-9′,10′-enyl-2-methoxy-GPC, and *rac*-1-hexadecyl-2-methoxy-GPC were purchased from R. Berchtold, Biochemisches Labor, Mattenhofstrasse 34, 3007 Bern, Switzerland. New England Nuclear (Boston, MA) catalytically tritiated *rac*-1-octadec-9′,10′-enyl-2-methoxy-GPC to form *rac*-1-[9′,10′-^{3}H]octadecyl-2-methoxy-GPC (8.2 mCi/μmol), and the latter was purified to 94.5% by thin-layer chromatography. Labeled and unlabeled 1-octadecyl-2-methoxy-glycerols were prepared from the labeled or unlabeled phospholipids by Vitride reduction (22).

1-Octadecylglyerol and 1-hexadecylglycerol were purchased from Foxboro-Analabs (North Haven, CT) and were determined to be 99.4 and 97.2% pure, respectively, by high-performance liquid chromatographic analysis of the dibenzoate derivatives (23). A preparation of 1-octadec-9′,10′-enylglycerol (selachyl alcohol) was originally obtained some years ago from Western Chemical Industries, Ltd. (Vancouver, British Columbia, Canada); the ether-linked moieties of isopropylidine derivatives of this selachyl alcohol preparation were determined by gas-liquid chromatography (24) to be 85.5% 18:1, 0.3% 18:0, 11.5% 16:1, and 0.4% 16:0. The 1-octadec-9′,10′-enylglycerol was tritiated by New England Nuclear via catalytic reduction. The radiolabeled product (1-[9′,10′-^{3}H]octadecylglycerol) after purification (98%) by preparative thin-layer chromatography [hexane:diethyl ether:methanol:acetic acid (70:30:5:1)] had a specific activity of 29.7 mCi/μmol. 1-[1′-^{14}C]Hexadecylglycerol (4.7 Ci/mol) purchased from New England Nuclear was also purified (94.2%) by thin-layer chromatography in the above solvent system.

1-[1′,2′-^{3}H]Alkyl-2-acetyl-GPC (45 Ci/mmol) with a radiopurity of 97% was prepared by M. Blank from our laboratories according to published methods (25). Labeled and unlabeled 1-alkyl-2-lyso-GPC were synthesized as described previously (8). The following materials were purchased: *N,N*-bis(2-hydroxyethyl)glycine (bicine) buffer, glutathione, and 1-alkyl-2-acetyl-GPC (PAF) (Sigma Chemical Co., St. Louis, MO); Pte·H_4-HCl (Regis Chemical Co., Morton Grove, IL); $(NH_4)_2SO_4$, enzyme grade (Schwarz-Mann Research Laboratories, Orangeburg, NY); propylene glycol (J. T. Baker Chemical Co., Phillipsburg, NJ); Vitride [$NaAlH_2(OCH_2CH_2OCH_3)_2$] (Eastman Organic Chemicals, Rochester, NY); and catalase (Boehringer-Mannheim, Indianapolis, IN). Lipid standards were obtained from Sigma except for phosphatidic acid (Serdary Research Laboratory, London, Ontario, Canada), tetradecyl aldehyde (Pfaltz & Bauer, Inc., Stamford, CT), and oleoyl alcohol (Foxboro-Analabs, North Haven, CT).

Cleavage of *rac*-1-[9′,10′-^{3}H]Octadecyl-2-methoxy-GPC in Cell Cultures. The HL-60 cells (26), obtained from Dr. R. C. Gallo, National Cancer Institute (Bethesda, MD), and K562 cells (27), provided by Dr. C. B. Lozzio, University of Tennessee (Knoxville), were cultured in RPMI Medium 1640, penicillin (100 units/ml), fungizone (100 μg/ml), 2 mM L-glutamine, and 20% heat-inactivated fetal bovine serum that was from Grand Island Biological Co. (Grand Island, NY). MDCK cells were obtained from the American Type Culture Collection (Rockville, MD) and human PMNs [isolated as described previously (8, 28) from normal peripheral blood (Plasma Alliance, Knoxville, TN)] were maintained in Dulbecco's modified essential medium, penicillin, fungizone, glutamine, and 10% fetal bovine serum. Except for the PMN, 1×10^7 cells in 10 ml medium were incubated at 37°C, 5% CO_2-95% air, for 24 h with 0.5 μM *rac*-1-[9′,10′-^{3}H]-octadecyl-2-methoxy-GPC (2 μCi/nmol) in 0.25% ethanol (final concentration). We incubated 3×10^7 PMN/10 ml medium under identical conditions, since PMN are smaller compared to HL-60 cells in size, on both a light microscopic basis and their content of protein (see "Results"). After cells were harvested and washed three times with 5 ml phosphate-buffered saline, aliquots of cells were assayed for radioactivity and the viable number of cells determined (trypan blue dye excluding cells; see Ref. 29). Total lipids were extracted from the cell suspensions by the procedure of Bligh and Dyer (30), except that the methanol contained 2% acetic acid; an aliquot of this extract was assayed for radioactivity. Neutral lipid species were fractionated on Silica Gel G layers developed in hexane:diethyl ether:methanol:acetic acid (70:30:5:1) and the radioactivity determined in 2-mm zonal scrapings of the silica gel layer (31). The phospholipid classes (lysophosphatidylcholine, phosphatidylcholine, sphingomyelin, phosphatidylserine, and phosphatidylethanolamine) were separated on Silica Gel H layers developed in chloroform:methanol:acetic acid:water (50:30:8:6); phosphatidylinositol and phosphatidic acid were resolved using a chloroform:methanol:ammonium hydroxide (60:35:8) solvent system.

The lipid products formed after treatment of an aliquot of the total cellular lipids by Vitride reduction (22) are 1-alkylglycerols and fatty alcohols (derived via reduction of acyl moieties); all other ester linkages are also removed (*e.g.*, phosphocholine at the *sn*-3 position). Radioactivity associated with the fatty alcohol fraction after the Vitride reduction is therefore proportional to the activity of the alkyl cleavage enzyme, since radioactivity originally incorporated into fatty alcohols or acyl moieties had to be derived from the *O*-alkyl group of the substrate. The Vitride-reduced lipids were separated on Silica Gel G layers developed in hexane:diethyl ether:methanol:acetic acid (70:30:5:1).

Pte·H_4-dependent Monooxygenase Assay. Activity of the microsomal alkyl cleavage enzyme was determined essentially as described previously (13, 18, 32). The incubation mixture contained 5 mM glutathione, 5 mM $(NH_4)_2SO_4$, 1 mM Pte·H_4, 100 mM bicine-NaOH (pH 8.8), 150 μM 1-[9′,10′-^{3}H]octadecylglycerol (30 μCi/μmol; in 30 μl propylene glycol), 50 units catalase, and 10 to 30 μg microsomal protein. Control incubations for residual cleavage activity were performed in the absence of Pte·H_4. A final volume of 0.5 ml was incubated for 5 min at 37°C and the reaction was terminated by lipid extraction. Lipid products (comigrating with authentic fatty aldehyde, fatty alcohol, and fatty acid) were isolated chromatographically and then analyzed by liquid scintillation assay.

Livers from CDF male rats were homogenized in a Potter-Elvehjem vessel fitted with a Teflon pestle. Homogenates of cultured cells were obtained by suspending the phosphate-buffered saline-washed cells in 10 mM Tris-HCl (pH 7.4)/1 mM EDTA (33) and then successively freezing them in liquid nitrogen followed by thawing (37°C) three times. Microsomal suspensions were prepared by conventional differential centrifugation (34) after adjusting the samples to 0.25 M sucrose/10 mM Tris (pH 7.4)/1 mM EDTA. The microsomal fraction was washed once in sucrose/Tris/EDTA, centrifuged, and finally resuspended in 0.25 M sucrose/10 mM Tris. Protein content was measured by the method of Lowry *et al.* (35).

Assessment of Alkyl Phospholipid Analogue Cytotoxicity. Details of the methodology have been reported previously in our earlier studies of the cytotoxic properties of numerous alkyl phospholipid analogues in cultured cells (8, 28). In the present investigation, cells were preincubated for 24 h in serum-free RPMI 1640 medium [containing penicillin (100 units/ml), fungizone (100 μg/ml), 2 mM L-glutamine, porcine insulin (0.12 units/ml), transferrin (5 μg/ml), and fatty acid-free fraction V bovine serum albumin (200 μg/ml)] before initiating the 24-h incubations containing varying concentrations of 1-hexadecyl-2-methoxy-GPC. The concentration of lipid that destroys 50% of the cells [viability determined by trypan blue dye exclusion (29)] after a 24-h incubation period was defined. Release of lactic acid dehydrogenase activity into the media (due to cell disruption; see Refs. 36 and 37), and depression of the [^{3}H]thymidine incorporation into cellular nucleic acids (data not presented) under identical conditions have also been used to verify the cytotoxic responses.

Radiolabeling of Cells and Autoradiography. HL-60, K562 cells (3×10^6), or PMNs (9×10^6) were incubated with 0.5 μM *rac*-1-[9′,10′-^{3}H]-octadecyl-2-methoxy-GPC (10 μCi) in 2 ml serum-free media for 20 h. In a separate experiment, 3×10^6 HL-60 cells were incubated with 0.5 μM (10 μCi) 1-[1′,2′-^{3}H]alkyl-2-acetyl-GPC for 20 h. The cells were

recovered, washed three times with phosphate-buffered saline, and resuspended in 0.3% bovine serum albumin/saline. Aliquots were smeared on 1- x 3-inch microscope slides, dried, fixed with 4% formaldehyde/phosphate-buffered saline for 1 h at 4°C, rinsed, and dried. The slides were coated (45°C) with Kodak nuclear track emulsion (NTB3 diluted 1:1 with distilled water). Samples were exposed to the emulsion in a desiccating chamber for 5 to 9 days, developed in Kodak D-19, and the cells were then stained with Gill's hematoxylin. An estimate of the distribution of radiolabel was obtained for each experiment by counting 10 cells in each of 40 randomly selected fields on the smears; the distribution pattern in individual cells was categorized as (*a*) uniformly labeled, (*b*) peripherally labeled, or (*c*) unlabeled. All cells having a number of grains higher than background (which was variable) were placed in either category 1 or 2; however, no estimate was made of the number of grains per cell.

RESULTS AND DISCUSSION

Cytotoxicity of 1-Hexadecyl-2-methoxy-GPC. Table 1 compares the cytotoxic responses of four selected cell types to the unnatural alkyl phospholipid analogue, *rac*-1-hexadecyl-2-methoxy-GPC. The two indices of cytotoxicity represent the concentrations of lipid required to destroy 50% of the cells or cause a 2-fold increase over control values of lactic acid dehydrogenase activity in the medium after incubating the cells for a 24-h period (8, 28). Sensitivity of human HL-60 cells and the relative insensitivity of PMN to cytotoxic alkyl phospholipid analogues have been reported previously (28). The response of HL-60 cells to incubations for short time periods with the 1-hexadecyl-2-methoxy analogue has also been characterized in earlier experiments (8). A differential response of K562 and HL-60 leukemia cells to the cytotoxic alkyl phospholipid, 1-octadecyl-2-methoxy-GPC, was first demonstrated by Tidwell *et al.* (21). The sensitivity of HL-60 cells and resistance of K562 cells to the 1-hexadecyl-2-methoxy analogue is confirmed in Table 1 where a 20-fold greater concentration of the lipid is needed to destroy 50% of the K562 cells.

Although both human cell lines are leukemic and possess similar generation times (24 h), they are distinguished as follows: (*a*) K562 cells are derived from patients with chronic myelogenous leukemia, whereas HL-60 cells are from patients with the acute form of the disease; (*b*) K562 cells are characterized as undifferentiated promyeloblasts resembling bone marrow stem cells (38, 39), which contrasts with the HL-60 cells, a more mature type of promyeloid cell (26); and (*c*) HL-60 cells can be induced to differentiate to macrophage-like cells by 12-*O*-tetradecylphorbol-13-acetate (40) as opposed to the K562 cells, which are resistant (39); therefore, it may be possible for the cytotoxic alkyl phospholipid analogues to initiate their antineoplastic actions on the basis of their ability to induce differentiation of leukemic cells as proposed by Honma *et al.* (11, 12). These investigators have reported that 1-octadecyl-2-methoxy-GPC can induce mouse myeloid leukemia cells to differentiate to macrophage-like cells at low lipid concentrations that do not affect normal mouse bone marrow cells. The MDCK cell line retains numerous characteristics of normal, differentiated cells (41) and this is further illustrated by their lack of response to the cytotoxic lipid (Table 1).

Table 1 *Cytotoxicity of 1-hexadecyl-2-methoxy-GPC and the microsomal activities of the Pte·H₄-dependent alkyl cleavage enzyme in CDF rat liver, human leukemia cells (HL-60 and K562), human PMNs, and MDCK cells*

Cell or tissue	Cytotoxicity[a] LC_{50}[b] (μM)	Cytotoxicity[a] Lactic acid dehydrogenase release[c] (μM)	Alkyl cleavage activity[d] (pmol/min/μg protein)
Rat liver			24.4 ± 3.0 (4)
K562	28.0 ± 6.0[e] (3)[f]	26.0 ± 3.0 (3)	0.22 ± 0.06 (6)
HL-60	1.5 ± 0.3 (8)	1.2 ± 0.1 (8)	0.25 ± 0.08 (10)
PMN	78.0 ± 2.0 (4)	56.0 ± 12.0 (4)	0.34 ± 0.20 (3)
MDCK	106.0 ± 6.0 (3)	99.0 ± 9.0 (3)	0.37 ± 0.21 (8)

[a] Cells were cultured in serum-free medium for cytotoxicity studies.
[b] LC_{50}, concentration required to kill 50% of the cells after a 24-h incubation.
[c] Concentration required to produce a 2-fold increase in lactic acid dehydrogenase activity above control values after a 24-h incubation.
[d] Activity is based on cleavage of 1-[9′,10′-^{3}H]octadecylglycerol at a saturating concentration of 150 μM.
[e] Mean ± SD.
[f] Numbers in parentheses, number of experiments.

Microsomal Pte·H₄-dependent Alkyl Cleavage Enzyme Activity. In attempting to explain the diverse responses of normal and neoplastic cells to the unnatural cytotoxic alkyl phospholipid analogues, Andreesen *et al.* (10) proposed that the alkyl cleavage enzyme is a primary factor in their selective cytotoxic mechanism toward cancer cells; thus, the inherently low cleavage activity of tumor cells (13) in comparison to normal cells would permit the unnatural alkyl phospholipid analogues to reach a concentration sufficient to inhibit vital cell functions and ultimately lead to cell death (10). However, previous investigations of the substrate specificity of rat liver microsomal alkyl cleavage enzyme (16) indicated that the unnatural cytotoxic alkyl phospholipids probably would not serve as an efficient substrate for the enzyme from either normal or tumor cells since an *sn*-2 lysoglycerolipid structure appears to be an essential feature of phospholipid substrates utilized by this enzyme.

To evaluate the role of the Pte·H_4-dependent alkyl cleavage enzyme, we assayed its activity in microsomes from cells that are sensitive (HL-60) and insensitive or resistant (K562, PMN, and MDCK) to cytotoxic 1-hexadecyl-2-methoxy-GPC (Table 1). Based on an earlier hypothesis (10) relating the low activity of the alkyl cleavage enzyme in cancer cells (13) to the accumulation of the unnatural alkyl phospholipids in these cells, the activity of the microsomal alkyl cleavage enzyme from the highly sensitive HL-60 cells would be expected to be much lower than in nonresponsive cell types; however, we found that the Pte·H_4-dependent alkyl cleavage activity in HL-60 cell microsomes did not differ significantly ($P < 0.05$) from that found in K562, human PMN, or MDCK cells.

The microsomal alkyl cleavage enzyme in rat liver has been used as a reference of comparison for the cleavage activity in cultured cells. Although the alkyl cleavage enzyme has been the focus of a number of previous investigations (13, 18, 20, 32), only Ishibashi and Imai (42) have reported K_m and V_{max} values for 1-hexadecylglycerol (660 μM and 29 pmol/min/μg protein, respectively). Therefore, since 1-[^{3}H]octadecylglycerol was used in the present assays, it was necessary to characterize the reaction kinetics for this substrate. The enzyme reaction was linear with time for 30 min and linear with protein up to 30 μg using the rat liver microsomal preparation. Other cellular fractions (12,000 × *g* pellet and the 100,000 × *g* supernatant) from rat liver or the four cell types did not possess a specific activity greater than 10% of that found in the microsomal fraction from the different cells. The K_m value for 1-octadecylglycerol was 72 ± 1 (SD) μM with a V_{max} value of 41 ± 3 pmol/min/μg protein when liver microsomes were the source of the enzyme; the specific activity of the alkyl monooxygenase was 24.4 ± 3.0 pmol/min/μg protein at a saturating concentration of 150 μM 1-octadecylglycerol (Table 1).

Substrate Specificity of the Alkyl Cleavage Enzyme. Since rat liver microsomes are a rich source of the Pte·H_4-dependent alkyl monooxygenase, we used this enzyme source to compare

the substrate specificity of the alkyl cleavage enzyme for 1-alkylglycerols and 1-octadecyl-2-methoxy-GPC (Table 2). Cleavage of the radiolabeled alkyl group of 1-hexadecylglycerol and 1-octadecylglycerol was much greater than for the *rac*-1-octadecyl-2-methoxy-glycerol or 1-alkyl-2-lyso-GPC; thus, substitution at either the 2- or 3-position of the 1-alkylglycerol decreases the cleavage activity by greater than 80%. When either the acetyl or methoxy group is present at the 2-position in an alkyl phospholipid, cleavage of the alkyl moiety did not occur; moreover, the microsomal enzyme derived from the four cell types (HL-60, K562, PMN, and MDCK) also had a markedly reduced capacity (compared to the rat liver enzyme) to cleave the ether-linked group of 1-[9′,10′-^{3}H]octadeeylglycerol (Table 1). The inherent low activity of the alkyl cleavage enzyme in the four cell types together with the finding that 1-octadecyl-2-methoxy-GPC failed to serve as a substrate indicates that the 2-methoxy phospholipid analogue could not be metabolized by the alkyl cleavage enzyme in these cells.

Cleavage of the Alkyl Ether Bond in Intact Cells. Extrapolation of enzymatic results to intact cells can often be misleading; therefore, we investigated the possibility that the alkyl moiety of 1-octadecyl-2-methoxy-GPC might be cleaved in cultures of four cell types that exhibit varying responses to the selective antineoplastic analogues. With *rac*-1-[9′,10′-^{3}H]octadecyl-2-methoxy-GPC as a substrate, a concentration of 0.5 μM was selected since the 2-methoxy analogue elicits cytotoxic effects toward HL-60 cells under these conditions within a 24-h incubation (approximately 20% of the cells are killed) but does not significantly affect the viability of the other three cell types.

Total recoveries of the radiolabel after lipid extraction was 89 ± 3% in HL-60, K562, and MDCK cells, but only 74 ± 7% in PMNs. Association of the radiolabeled 1-octadecyl-2-methoxy-GPC with the cytotoxically sensitive HL-60 cells was significantly greater than in any of the other cells (Table 3). This difference is even more apparent when the cellular radioactivity of total lipids is calculated on a protein basis. The latter is due to differences in the protein content of the cells: 206 ± 49 μg protein/10^6 HL-60 cells compared to 320 ± 39, 60 ± 10, and 405 ± 10 μg protein/10^6 K562, PMN, and MDCK cells, respectively. The uptake of radioactivity as used here refers to the retention of tritiated lipids with cells (intracellularly or adherence to the surface membrane) after washing three times with phosphate-buffered saline. In a separate experiment where the dose of the 1-octadecyl-2-methoxy analogue expressed no cytotoxic effects on any of the cell types (12.4 nM; 10 μCi), the cellular uptake of radioactivity was essentially identical to that shown in Table 3. The close association of the 2-methoxy

Table 2 *Substrate specificity of Pte·H_4-dependent alkyl cleavage enzyme in rat liver microsomes*

Lipid (20 μM)	% control
1-[1′-^{14}C]Hexadecylglycerol (3)[a]	186 ± 16[b]
1-[9′,10′-^{3}H]Octadecylglycerol[c] (5)	100 ± 10
rac-1-[9′,10′-^{3}H]Octadecyl-2-methoxy-glycerol (4)	17 ± 5.4
1-[1′,2′-^{3}H]Alkyl-2-lyso-GPC (3)	16 ± 2.6
1-[1′,2′-^{3}H]Alkyl-2-acetyl-GPC (3)	1.8 ± 0.2
rac-1-[9′,10′-^{3}H]Octadecyl-2-methoxy-GPC (4)	0.5 ± 0.2

[a] Numbers in parentheses, number of experiments.
[b] Mean ± SD.
[c] Specific activity of the 100% control (1-octadecylglycerol) was 8.3 ± 0.9 pmol/min/μg microsomal protein.

Table 3 *Association of radioactivity with normal and leukemic cells incubated with rac-1-[9′,10′-^{3}H]octadecyl-2-methoxy-GPC*

Approximately 10^7 cells/10 ml 20% serum-RPMI 1640 medium were incubated with 0.5 μm (10 μCi) *rac*-1-[9′,10′-^{3}H]octadecyl-2-methoxy-GPC for 24 h. Results are mean ± SD.

Phospholipid analogue associated with cells	Cell type			
	HL-60 (7)[a]	K562 (7)	PMN (5)	MDCK (4)
pmol/10^6 cells[b]	377 ± 20	188 ± 35[c]	45 ± 14[c]	175 ± 13[c]
pmol/mg protein	1750 ± 170	540 ± 64[c]	720 ± 160[c]	380 ± 33[c]

[a] Numbers in parentheses, number of experiments.
[b] Based on molecular weight of 1-octadecyl-2-methoxy-GPC.
[c] Significantly different from those found for HL-60 cells based on Student's *t* test at $P < 0.05$.

Table 4 *Distribution of radioactivity in lipids of cells incubated with rac-1-[9′,10′-^{3}H]octadecyl-2-methoxy-GPC*

Neutral lipids were separated on Silica Gel G layers developed in hexane:diethyl ether:methanol:acetic acid (70:30:5:1). Phospholipids were separated on Silica Gel H layers developed in chloroform:methanol:acetic acid:water (50:30:8:6) and chloroform:methanol:ammonium hydroxide (60:35:8). Results are mean ± SD. Distribution of radioactivity was based on 2-mm zonal scans of the chromatograms. No radioactivity cochromatographed with lysophosphatidylcholine, sphingomyelin, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, fatty acid, or fatty alcohol standards.

Lipid class	% of total lipids for			
	HL-60 (4)[a]	K562 (3)	PMN (3)	MDCK (4)
1-Alkyl-2-methoxy-GPC	93.0 ± 6.3	92.0 ± 2.1	87.0 ± 7.0	97.0 ± 3.8
Choline glycerophospholipids	4.0 ± 1.8	4.8 ± 2.3	8.2 ± 2.4[b]	3.5 ± 1.1
Alkylglycerols	0	0	1.4 ± 0.8	0
1-Alkyl-2-methoxy-glycerol	1.1 ± 0.5	0	1.4 ± 0.8	0
Solvent front area	1.5 ± 0.9	3.0 ± 1.4	0.6 ± 0.3	0

[a] Numbers in parentheses, number of experiments.
[b] Significantly different from that found for HL-60 cells based on the Student's *t* test at $P < 0.05$.

Table 5 *Distribution of tritium in Vitride-reduced products from total lipids of normal and leukemic cells after incubation with rac-1-[9′,10′-^{3}H]octadecyl-2-methoxy-GPC*

Vitride-reduced lipids from cells after incubation for 24 h with *rac*-1-[9′,10′-^{3}H]octadecyl-2-methoxy-GPC were separated on Silica Gel G layers developed in hexane:diethyl ether:methanol:acetic acid (70:30:5:1). Radioactivity distribution was based on 2-mm zonal scans of the chromatograms and corrected for background and radiopurity (95.2%) of the Vitride-reduced 1-[^{3}H]octadecyl-2-methoxy-GPC.

Lipid class	% of total lipid radioactivity for cell type			
	HL-60 (4)[a]	K562 (3)	PMN (3)	MDCK (4)
Unmetabolized substrate				
1-Alkyl-2-methoxyglycerol	93.0 ± 6.0[b]	91.0 ± 6.0	88.0 ± 5.0	95.0 ± 5.0
Metabolites				
Origin	0	1.3 ± 0.2	2.4 ± 0.6	0.9 ± 0.3
Alkylglycerol-containing lipids	3.8 ± 0.4	0.6 ± 0.1[c]	5.9 ± 2.5	0
Fatty alcohol derived from acyl moieties	2.9 ± 0.2	3.5 ± 0.8	2.3 ± 0.5[c]	3.7 ± 0.4[c]
Solvent front area	0	3.6 ± 1.0	1.4 ± 0.8	0

[a] Numbers in parentheses, the number of experiments.
[b] Mean ± SD.
[c] Significantly different from those found for the HL-60 cells based on the Student's *t* test at $P < 0.05$.

Table 6 *Distribution of radiolabel in cultured cells treated with rac-1-[^{3}H]-octadecyl-2-methoxy-GPC or ^{3}H-labeled PAF*

Data represent a quantitative evaluation of the silver grains present for each experimental condition represented in Fig. 1. Values are the average of the analysis of 40 random fields viewed at ×200; 10 cells/field were counted.

Cell type/radiolabel	% distribution		
	No label	Uniform	Peripheral
HL-60/^{3}H-labeled 2-methoxy analogue (Fig. 1, *a* and *b*)	8	30	62
K562/^{3}H-labeled 2-methoxy analogue (Fig. 1*c*)	46	23	31
PMN/^{3}H-labeled 2-methoxy analogue (Fig. 1*d*)	36	61	3
HL-60/^{3}H-labeled PAF (Fig 1*e*)	4	83	13

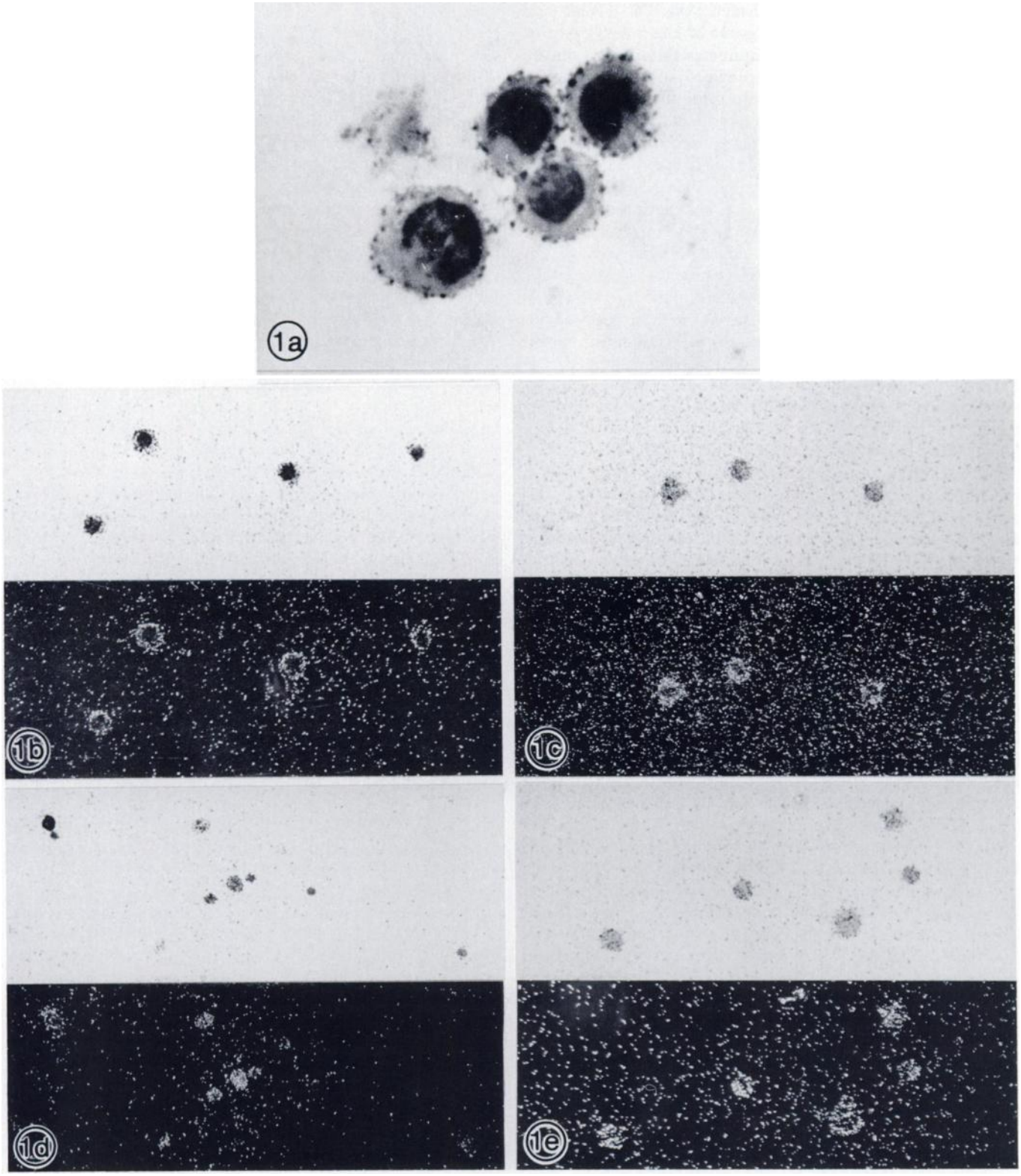

Fig. 1. Light microscopic autoradiographs of cell smears exposed for 20 h to ^{3}H-labeled phospholipids. *a* to *d*, cells incubated in serum-free medium with 10 μCi *rac*-1-[9′,10′-^{3}H]octadecyl-2-methoxy-GPC. *a*, HL-60 cells, × 2200. *b*, *upper section*, same as *a*, × 275; *lower section*, corresponding darkfield view. *c*, K562 cells at × 275 and corresponding darkfield view. *d*, PMN at × 275 and corresponding darkfield view. *e*, HL-60 cells incubated with 10 μCi 1-[1′,2′-^{3}H]alkyl-2-acetyl-GPC at × 275 and corresponding darkfield view. See "Materials and Methods" for details.

analogue with the HL-60 cells compared to the other cell types could relate to differences in the surface charge or specific receptors at the plasma membrane between sensitive and resistant cells. A greater electronegative charge on the cell surface of tumor cells in comparison to normal cells has been described (43, 44) and appears to be due to the presence and/or quantity of specific macromolecules. The 2-methoxy analogue might also interact with a lipophilic receptor on the surface of the

sensitive neoplastic cell, since specific receptors for PAF (45) and phorbol diesters (46) have been reported.

The extent of metabolism of the ^{3}H-labeled alkyl group of the 2-methoxy phospholipid analogue was determined on the basis of the radiolabeled products formed after incubation of the cells with *rac*-1-[9′,10′-^{3}H]octadecyl-2-methoxy-GPC. Eighty-seven to 97% of the radioactivity was still present as the unaltered 1-octadecyl-2-methoxy phospholipid analogue in all four cell types after the 24-h incubation (Table 4).

After thin-layer chromatography, the labeled neutral lipids gave distinct, reproducible peaks that corresponded to standards of 1-octadecyl-2-methoxyglycerol and 1-octadecenylglycerol. These metabolic products could be derived from the parent compound by a phospholipase C activity and by cleavage of the 2-methoxy moiety by some unknown enzymatic mechanism.

The quantity of radioactivity found in the fatty alcohol fraction (derived from labeled acyl groups or the aldehyde intermediate) after Vitride reduction of the total cellular lipids (Table 5) reflects the activity of the alkyl cleavage enzyme in intact cells since fatty acids, aldehydes, or alcohols are the only labeled products formed by the enzymatic reaction (20, 31). The amount of label in the fatty alcohol fraction of HL-60 cell lipids was lower (on a percentage basis) than that in MDCK cells but higher than in PMN lipids (Table 5); however, no causal relationship is evident between the sensitivity response of the four cell types to the cytotoxic phospholipid and the lack of alkyl cleavage. No significant differences were found in the amount of label associated with the 1-octadecyl-2-methoxyglycerol fraction after Vitride reduction of the lipids in any of the cell types after a 24-h incubation with the labeled 2-methoxy phospholipid analogue (Table 5). These results indicate that at the very most, only 5 to 12% of the 2-methoxy analogue was metabolized by the cells. From these observations we conclude that the antineoplastic property of the unnatural alkyl phospholipid analogues cannot be simply explained on the basis of the low alkyl cleavage enzyme activity found in most cancer cells.

Autoradiography of Cells Incubated with ^{3}H-labeled Phospholipids. Light microscopic observations of autoradiographic preparations demonstrated that the majority (61%; Table 6) of HL-60 cells treated for 20 h with 0.5 μM (10 μCi) *rac*-1-[9′,10′-^{3}H]octadecyl-2-methoxy-GPC were heavily labeled at the periphery, (Fig. 1*a*), suggesting retention of the radiolabeled 2-methoxy phospholipid analogue at, or just beneath, the plasma membrane. This halo of silver grains was even more pronounced when viewed under darkfield conditions (Fig. 1*b*). Compared to the HL-60 cells, the K562 cells and PMNs were categorized to a greater degree as either unlabeled (46 and 35.5%, respectively) or uniformly labeled (23 and 61%, respectively; Fig. 1 *c* and *d*; Table 6). Compared with HL-60 cell preparations, the PMN and K562 cells exhibited limited cellular labeling after exposure to the tritiated 2-methoxy phospholipid analogue (Fig. 1 *c* and *d*) and neither cell type had the peripheral ring of silver grains, which characterized the autoradiographs of the HL-60 cells. In contrast when HL-60 cells were incubated with 0.5 μM PAF (10 μCi 1-[1′,2′-^{3}H]alkyl-2-acetyl-GPC) for 20 h, the autoradiograms displayed primarily a uniform distribution of the silver grains (83% of cells; Fig. 1*e*; Table 6). This observation is consistent with the earlier metabolic data obtained by Cabot *et al.* (47) that demonstrated HL-60 cells rapidly converted labeled PAF to 1-alkyl-2-lyso-GPC and 1-alkyl-2-acyl-GPC.

Our data indicate that the low alkyl cleavage enzyme activity in tumor cells does not explain the antineoplastic action of the 2-methoxy phospholipid analogue. To begin with, the alkyl cleavage enzyme has been shown to have a low activity in normal PMN and MDCK cells as well as HL-60 cells even though they respond differently to the cytotoxic effects of the 2-methoxy analogue. In addition, 1-octadecyl-2-methoxy-GPC is not a substrate for the cleavage enzyme; moreover, morphological data indicate that only a relatively small amount of the cytotoxic phospholipid penetrates the interior of cells where the alkyl cleavage enzyme is located.

The present investigation also demonstrates that the sensitive human leukemic cells show a preferential sequestering of the antineoplastic 2-methoxy phospholipid analogue (Table 3) and that this unnatural phospholipid analogue appears to be enriched in the plasma membrane (Fig. 1 *a* and *b*). We have recently documented the localization of 1-octadecyl-2-methoxy-GPC at the surface of HL-60 cells in experiments where the plasma membranes were isolated by ultracentrifugation of the subcellular organelles in a Percoll gradient (48). Our data further substantiate the finding of Arnold *et al.* (49) who found that when *rac*-1-[^{3}H]octadecyl-2-methoxy-GPC was administered intravenously to rats more of the radioactivity accumulated in tumor tissues than in normal tissues. Enrichment of the unnatural 2-methoxy phospholipid analogue in the surface membranes of the cell could inhibit vital cell functions such as lipid metabolic enzymes involved in the assembly of membranes (50), Ca^{2+}-phospholipid dependent protein kinase (51), sialyltransferase (52), and/or the transport of required nutrients across the plasma membrane (53) that could ultimately lead to cell death.

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