Biochemical Pharmacology, Vol. 45, No. 12, pp. 2435–2439, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

UNIDIRECTIONAL MEMBRANE UPTAKE OF THE ETHER LIPID ANTINEOPLASTIC AGENT EDELFOSINE BY L1210 CELLS

ERIC E. KELLEY, EDWARD J. MODEST and C. PATRICK BURNS*

Department of Medicine, The University of Iowa College of Medicine, Iowa City, IA 52242; and Department of Biochemistry, Boston University Medical School, Boston, MA 02118, U.S.A.

(*Received* 24 *November* 1992; *accepted* 26 *February* 1993)

Abstract—We have studied the cellular uptake of edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; ET-18-OCH_3), a membrane active anticancer drug of the ether lipid family, by L1210 murine leukemia cells. Initial unidirectional linear uptake velocity was 1.1 nmol/min per 2×10^6 cells; at about 30 min it reached a steady-state phase of accumulation of approximately 5 nmol/2×10^6 cells. Concentration studies indicated no saturation kinetics from 0 to 40 μM. Studies with metabolic inhibitors displayed no energy dependence. There was no effect of chloroquine, monensin or cytochalasin B, which are known inhibitors of endocytosis. The inhibitory effect of lower temperature on uptake was moderate in extent and compatible with passive diffusion. There was no efflux of drug from preloaded cells which indicates intense binding of incorporated drug to cells. In human serum, edelfosine bound to several protein components, primarily high density lipoprotein and albumin, and this may explain why cellular uptake was slowed considerably by the presence of serum or albumin in the incubation medium. We conclude that the lipophilic ether lipid derivative edelfosine is taken up by passive diffusion by the L1210 cell. It is tightly bound to cellular structures, probably by insertion into the membrane lipid bilayer.

The ether lipids, analogs of lysophosphatidylcholine, are an interesting new class of antineoplastic agents. They localize in cellular membranes [1, 2] and have no known direct interaction with DNA. The characteristics of the cellular accumulation of the drug have been studied by Bazill and Dexter [3], and they concluded that one component of uptake is endocytosis. In their studies, uptake of tritiated edelfosine by drug-sensitive WEHI-3B leukemia cells was reduced by inhibitors of endocytosis (chloroquine, monensin, and vinblastine) and by sodium fluoride. However, uptake was nonsaturable at high concentrations, and it was not affected by sodium azide. Storch and Munder [4] reported that edelfosine uptake by MethA fibrosarcoma and other drug-sensitive cell lines is nonsaturable and unaffected by metabolic inhibitors, but slowed by cytochalasin B and dipyridamole. However, both studies were performed using experimental time points at hourly intervals; therefore, the results measured gradual drug accumulation, but not initial unidirectional uptake. In addition, those studies were carried out in the presence of 10% fetal bovine serum. The characteristics of initial uptake at early time points have not been reported. We carried out our studies at early and short time intervals so that unidirectional drug uptake was not complicated by drug efflux, metabolism or intracellular binding. The use of a serum-free transport medium allowed us to eliminate the confounding effect of binding to serum components. Since ether lipids are lipid-soluble agents and phospholipid derivatives, we anticipated that they might be incorporated directly into the plasma membrane by passive diffusion. As analogs of membrane phospholipids, they represent a new approach to cytotoxicity of neoplastic cells; therefore, the kinetic features of their cellular uptake and of binding to human serum are of particular interest.

MATERIALS AND METHODS

L1210 murine leukemia cells were grown in suspension culture at 37° in medium consisting of RPMI 1640 (Grand Island Biological Co., Grand Island, NY) and heat-inactivated 10% fetal bovine serum (Sigma Chemical Co., St Louis, MO) in a humid atmosphere of 5% CO_2:95% air. Cells were harvested during the exponential growth phase and resuspended in balanced salt solution (BSS†, 132 mM NaCl, 16 mM Na_2HPO_4, 5 mM KCl, 1 mM $MgSO_4$, 5.6 mM glucose, pH 7.4) for all uptake studies.

Unlabeled edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; ET-18-OCH_3) was provided by Medmark Pharma GmbH (Dr. R. Nordström, Munich, Germany). [9,10-$^3H_2$(N)]-1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine ([^{3}H]edelfosine; sp. act. 56 Ci/mmol; purity >98% by TLC) was provided by Dr. Steven Wyrick, University of North Carolina, Chapel Hill, NC [5]. It was stored in absolute ethanol until immediately before use, mixed with unlabeled edelfosine (some experiments) and added to BSS to a final ethanol concentration of ≤0.4%. This concentration of ethanol had no effect on cell viability.

* Corresponding author: C. Patrick Burns, M.D., Department of Medicine, University Hospitals, Iowa City, IA 52242. Tel. (319) 356-2038; FAX (319) 353-8383.

† Abbreviations: BSS, balanced salt solution; and HDL, high density lipoprotein.

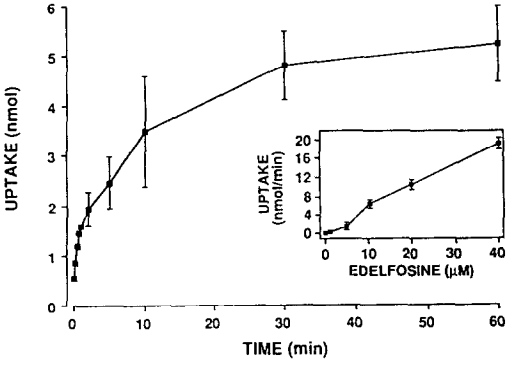

Fig. 1. Time course of edelfosine uptake at 37°. L1210 cells were incubated with 5 μM [^{3}H]edelfosine in BSS. At the conclusion of the time intervals, the cells were rapidly separated by centrifugation, and the radioactivity of the cell pellet was determined. Each value is the mean ± SEM of three separate experiments. The equation of the linear regression analysis for the first minute was $Y = 1.0896X + 0.5882$, $r = 0.991$. There was no appreciable cell death even after 2 hr at this concentration. The inset shows the effect of concentration of drug on uptake at 60 sec during the initial linear phase. Uptake is expressed in nmol/2 × 10^6 cells. Values are means ± SEM of three separate experiments.

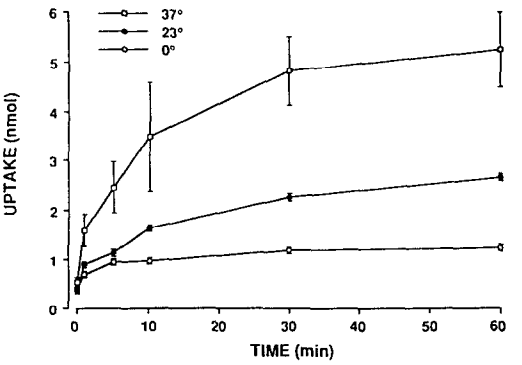

Fig. 2. Effect of temperature on edelfosine uptake. Cells were incubated with 5 μM drug at the three temperatures shown. Uptake is expressed in nmol/2 × 10^6 cells. Values are means ± SEM of three separate experiments.

Uptake studies were performed by a modification of techniques previously described from our laboratory [6–10]. Briefly, uptake studies were carried out in 15-mL conical centrifuge tubes containing 4 × 10^6/mL L1210 cells in 4 mL BSS. [^{3}H]Edelfosine was added after temperature equilibration, and experiments were terminated after incubation by quickly transferring an aliquot of the samples to 1.5-mL Eppendorf centrifuge tubes containing 0.3 mL *n*-butyl phthalate:corn oil (3:1, v/v) layered beneath 0.5 mL BSS at 0°. Rapid separation of the cells from the incubation medium was accomplished by immediate centrifugation at 16,000 *g* for 2 min in an Eppendorf 5412 centrifuge. The supernatant containing the unincorporated ether lipid was immediately aspirated, and the tip of the 1.5-mL tapered microfuge tube containing the pellet was severed and placed in a scintillation vial containing 0.5 mL Tissue Solubilizer (Research Products International Corp., Mount Prospect, IL). After 2 hr, 5.0 mL Budget Solve scintillation fluid (Research Products International Corp.) was added, the samples were vortexed, and radioactivity was determined using a Beckman LS3133T scintillation counter.

Efflux studies were performed using L1210 cells preloaded for 45 min at 37° with 5 μM [^{3}H]edelfosine. The cells were then rapidly chilled, washed at 0°, and resuspended in BSS at 37° to initiate efflux. Aliquots removed at specified times were filtered through Whatman GC/C glass microfiber filters, washed five times with BSS, dried, and counted for radioactivity.

The effects of metabolic inhibitors on drug uptake were studied by preincubating the cells with the inhibitor in BSS for the specified times prior to a 1-min incubation with 5 μM [^{3}H]edelfosine. The effects of human serum and human albumin were ascertained by placing various concentrations of human serum or fatty acid-free albumin with cells and incubating for 1 min in medium containing 5 μM [^{3}H]edelfosine.

To study binding to serum components, freshly obtained human serum was incubated for 1 min with 1.9 μM [^{3}H]edelfosine, and then a 2-mL aliquot was placed on top of a 7-step KBr discontinuous gradient (d = 1.0117 to 1.215 g/mL) using a modification of a published method [11]. After centrifugation at 121,000 *g* for 48 hr, the layers were separated, and the amount of radioactivity in each was determined.

Data were evaluated for statistical significance using the unpaired Student's *t*-test, and standard regression analysis was performed on software of the Prophet system, General Clinical Research Centers Program, NIH.

RESULTS

Time course of edelfosine uptake. The kinetics of uptake of 5 μM [^{3}H]edelfosine is shown in Fig. 1. The initial rate of influx during the first 60 sec, measured as the slope of the regression analysis during the linear phase, was 1.1 nmol/min per 2 × 10^6 cells. Uptake leveled off at approximately 30 min and entered a near steady-state phase of total accumulation of approximately 5 nmol/2 × 10^6 cells, and this persisted for at least 60 min. After early rapid binding, the initial unidirectional uptake was approximately linear for 1 min. Accordingly, subsequent kinetic experiments were conducted using a time interval of 1 min to ensure that only unidirectional drug influx was measured.

Rapid association of the drug to the cell membrane occurred. Extrapolation of the time–course curve to the y-axis yielded a value of 0.59 nmol/2 × 10^6 cells. This temperature-independent nonspecific binding can also be estimated by observing uptake at 0° at as short a time point as technically possible. This

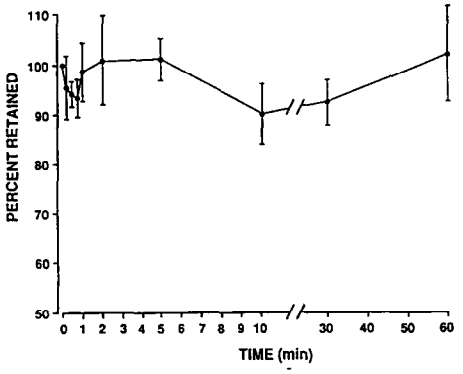

Fig. 3. Efflux of edelfosine in L1210 cells. Cells were incubated with 5 μM drug for 45 min at 37° for maximum preloading, and then were chilled to 0° during washing. Efflux at 37° was measured by determining residual radioactivity at the times shown. The 100% value at time zero was 1.21 ± 0.06 nmol. Values are means ± SEM of three separate experiments.

observation yielded a value of 0.54 nmol/2 × 10^6 cells, which is similar to the value calculated above.

Effect of concentration on uptake. The unidirectional influx of edelfosine at 60 sec was measured at various concentrations up to 20 μM (the maximal sublethal concentration at 60 sec determined by trypan blue dye exclusion) (Fig. 1 inset). From 0 to 20 μM there was approximate linearity ($r = 0.99$). Observations were also made at 40 μM and linearity was maintained; however, cell viability was only 4% at this high concentration. No saturable uptake was observed. The line passed through the origin, suggesting that there was no undetected high-affinity system that was saturated at the lowest experimental concentration.

Temperature sensitivity of edelfosine influx. The rates of influx and accumulation of the drug were different at the three temperatures studied (Fig. 2). At 37° uptake of the drug increased rapidly during the first 10 min and then slowed to a plateau of about 5 nmol/2 × 10^6 cells. Uptake was reduced at 23° as compared to 37°, reaching a steady-state level of approximately 50% of the values for 37°. Uptake was still lower at 0°, reaching a plateau about 50% lower than 23°. From these data, the Q_{10} can be estimated to be less than 2, which is compatible with, if not suggestive of, a diffusion process [12]. Early, unidirectional initial influx was explored at time points of less than 1 min, and the difference in unidirectional influx for the three temperatures, including the estimated maximum Q_{10} value, was consistent with passive diffusion.

Efflux of edelfosine. We examined the rate of efflux of edelfosine from L1210 cells that had been preloaded with drug (Fig. 3). The lack of appreciable efflux indicates tight irreversible association of drug with cellular structures.

Effects of metabolic inhibitors. The effects on initial uptake of prior incubation at 37° of L1210 cells with eight metabolic inhibitors are shown in Table 1. There was little or no effect of any inhibitor. In additional studies, three of these compounds were incubated with the cells for 1 hr prior to uptake studies. This longer incubation time failed to identify an inhibitory effect (chloroquine 500 μM, 80 ± 6% of control; monensin 10 μM, 93 ± 2%; sodium azide 10 mM, 90 ± 4%). Further testing was done with chloroquine, a known inhibitor of endocytosis. Six concentrations between 50 μM and 100 mM were studied and a 10-min preincubation was used. All failed to inhibit initial unidirectional uptake at 50 μM (86 ± 6% of control), 100 μM (92 ± 3%), 500 μM (99 ± 2%), 1 mM (100 ± 2%), 50 mM (109 ± 3%) and 100 mM (106 ± 4%).

Effects of serum on uptake and serum binding. The effects of both human albumin and human serum in BSS on edelfosine uptake are shown in Fig. 4. Human serum reduced uptake by approximately 50% at levels as low 0.5% serum. When human albumin was used in place of serum, uptake was inhibited in a manner similar, but not identical, to that of serum.

These results led us to explore the nature of the protein binding of edelfosine in serum. We incubated radiolabeled drug with freshly obtained human serum, and then carried out a serum lipoprotein separation by preparative ultracentrifugation (Fig. 5). Of total drug recovered (84%), the majority was associated with high density lipoprotein (HDL) or albumin.

DISCUSSION

Our data indicate that the initial unidirectional influx of the membrane active ether lipid edelfosine by L1210 cells is nonsaturable, energy independent and only moderately temperature sensitive. These are characteristics of passive diffusion. Edelfosine is a lipophilic drug of medium size (molecular weight = 524); molecules of this size are usually not taken up by endocytosis. Since the major determinants of diffusion are lipophilicity and molecular weight [12], it is not surprising that this compound associates with cell membranes by passive diffusion. Furthermore passive diffusion is a feasible mechanism to explain the fact that this compound localizes in the plasma membrane [1, 2]. Bazill and Dexter [3] reported that uptake of edelfosine by WEHI-3B and HL-60 leukemia cells is at least in part by endocytosis. They reported that edelfosine is taken up by both energy-independent and energy-dependent components. We found no evidence for energy dependence. There is a suitable explanation for the differences in their conclusions and those of our study. They studied drug uptake in the presence of serum; thus, the characteristics described could have been those of drug–protein complex uptake. Furthermore, the study of initial drug uptake to determine the mechanism requires early time points prior to the effects of intracellular drug accumulation, intracellular binding, metabolism and efflux. We carried out all experiments during a time within the linear phase of the time course to ensure that initial uptake velocity was measured. Bazill and Dexter did their studies at 1–3 hr, so they studied gradual drug accumulation rather than initial unidirectional uptake.

Table 1. Effects of metabolic inhibitors on [^{3}H]edelfosine influx by L1210 cells

Inhibitor	Inhibitor concentration (mM)	% of Control
NaF	10	99.4 ± 6.8
	20	97.6
KCN	0.1	91.8 ± 5.2
NaN_3	10	97.6 ± 4.0
2,4-Dinitrophenol	2	104.9 ± 8.6
Antimycin A	0.01	103.9 ± 9.7
Chloroquine	0.05	86.4 ± 6.0
	0.10	91.9 ± 3.2
Monensin	0.01	93.0 ± 1.7
Cytochalasin B	0.06	84.0 ± 0.3

Cells were preincubated with the inhibitor for 10 min (except for cytochalasin B, 60 min) at 37° prior to the influx study. A 5 μM concentration of [^{3}H]-edelfosine was added, and the 60-sec uptake was determined. Values are expressed as a percentage of influx in the absence of inhibitor and are the means ± SEM of determinations from three separate experiments. The inhibitor concentrations used had no effect on cell viability. The control values (each as cpm/2 × 10^6 cells) were: 10 mM, NaF 3410; 20 mM NaF, 3070; KCN, 3410; NaN_3, 3410; 2,4-dinitrophenol, 3190; antimycin A, 3190; 0.05 mM chloroquine, 3410; 0.1 mM chloroquine, 3410; monensin, 3370; and cytochalasin B, 4880.

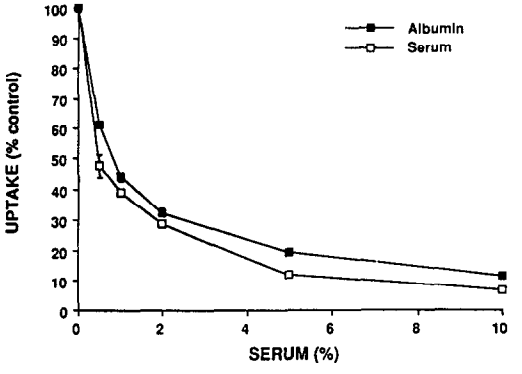

Fig. 4. Effect of human serum or human albumin on edelfosine uptake. L1210 cells were incubated with 5 μM drug for 1 min in the presence of freshly obtained human serum or fatty acid-free human albumin. Values, expressed as means ± SEM of three separate experiments, are the percentages of cells incubated in the absence of albumin and serum. The 100% control values were 4990 ± 120 cpm (albumin) and 5640 ± 280 cpm (serum).

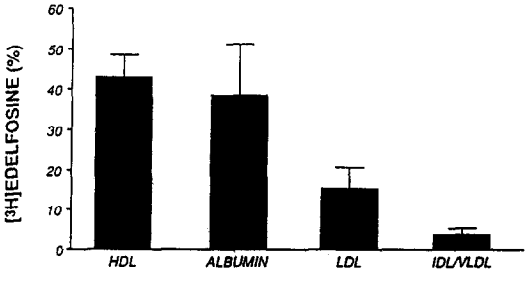

Fig. 5. Binding of edelfosine to human serum proteins. [^{3}H]Edelfosine (1.9 μM) was incubated for 1 min in freshly obtained human serum. An aliquot was placed on top of a 7-step KBr discontinuous gradient and centrifuged at 121,000 *g* for 48 hr. The layers were separated and the radioactivity in each was counted. Shown is radioactivity in each fraction as a percentage of total recovery. Values are means ± SEM of three separate experiments. Abbreviations: LDL, low density lipoprotein; and IDL/VLDL, intermediate density lipoprotein/very low density lipoprotein.

We have shown that edelfosine is bound to serum proteins, primarily HDL and albumin. Kötting *et al.* [13] also found substantial amounts of edelfosine bound to serum proteins other than albumin, but they reported that a majority of the drug (71%) was bound to albumin and only 6% to HDL. This difference from our study is likely due to the fact that they incubated human serum with high concentrations (100 μM) of edelfosine that cannot be attained in the bodies of rodents or humans. It seems likely to us that the HDL binding sites were overwhelmed, and excess drug then became bound to the more plentiful albumin. We used pharmacologic concentrations of 1.9 μM. Our studies are consistent with the binding of the structurally similar lysophosphatidylcholine, which was more avidly bound to the HDL than to albumin of monkey plasma [14]. From our data it can be concluded that protein binding reduces the extracellular concentration of free drug and cellular uptake of edelfosine. In this regard, previous work in our laboratory has shown that the addition of serum to the incubation medium will reduce the cytotoxicity of ilmofosine, a related ether lipid drug, in a concentration-dependent manner as measured in a

clonogenic assay [15], and this is likely related to reduced uptake. Serum protein binding of this drug impedes passive diffusion and reduces cytotoxicity in a manner similar to the binding of other hydrophobic compounds such as lysophospholipids and free fatty acids [16, 17].

Studies on endothelial and liver cells have shown that albumin binds reversibly to specific saturable sites on the cell surface and releases selected ligands without being internalized [18, 19]. It is also likely that HDL binds reversibly to high-affinity sites on extrahepatic cells such as fibroblasts and macrophages without being taken up [20]. HDL or albumin may anchor on the cell surface and deliver bound ether lipid gradually. This could explain, in part, the experiments done in the presence of 10% serum, which suggested uptake of ether lipids by endocytosis [3, 4].

Acknowledgements—This investigation was supported by Grants CA31526 and CA41314 awarded by the National Cancer Institute, Department of Health and Human Services and The Art Ehrmann Cancer Fund of the Fraternal Order of Eagles. Data analysis utilized the Prophet system, Grant RR59 from the General Clinical Research Centers Program, Division of Research Resources, NIH. We thank Glenna L. Fry, Michelle A. Waknitz and Dr. David A. Chappell for assistance with the lipoprotein separation technique.

REFERENCES

1. Hoffman DR, Hoffman LH and Snyder F, Cytotoxicity and metabolism of alkyl phospholipid analogues in neoplastic cells. *Cancer Res* **46**: 5803–5809, 1986.
2. van Blitterswijk WJ, Hilkmann H and Storme GA, Accumulation of an alkyl lysophospholipid in tumor cell membranes affects membrane fluidity and tumor cell invasion. *Lipids* **22**: 820–823, 1987.
3. Bazill GW and Dexter TM, Role of endocytosis in the action of ether lipids on WEHI-3B, HL60, and FDCP-mix A4 cells. *Cancer Res* **50**: 7505–7512, 1990.
4. Storch J and Munder PG, Increased membrane permeability for an antitumoral alkyl lysophospholipid in sensitive tumor cells. *Lipids* **22**: 813–819, 1987.
5. Wyrick SD, Surles JR, Morris-Natschke S, Piantadosi C and Modest EJ, Tritium labelled 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (ALP) and 1 - S - hexadecyl - 2 - O - ethyl - rac - thioglycero - 3-phosphocholine (thio-ALP). *J Labelled Compd Radiopharm* **27**: 269–275, 1989.
6. Burns CP, Luttenegger DG, Dudley DT, Buettner GR and Spector AA, Effect of modification of plasma membrane fatty acid composition on fluidity and methotrexate transport in L1210 murine leukemia cells. *Cancer Res* **39**: 1726–1732, 1979.
7. Burns CP and Dudley DT, Temperature dependence and effect of membrane lipid alteration on melphalan transport in L1210 murine leukemia cells. *Biochem Pharmacol* **31**: 2116–2119, 1982.
8. Burns CP and North JA, Adriamycin transport and sensitivity in fatty acid-modified leukemia cells. *Biochim Biophys Acta* **888**: 10–17, 1986.
9. Burns CP, Haugstad BN and North JA, Membrane transport of mitoxantrone by L1210 leukemia cells. *Biochem Pharmacol* **36**: 857–860, 1987.
10. Burns CP, Haugstad BN, Mossman CJ, North JA and Ingraham LM, Membrane lipid alteration: Effect on cellular uptake of mitoxantrone. *Lipids* **23**: 393–397, 1988.
11. Kelley JL and Kruski AW, Density gradient ultracentrifugation of serum lipoproteins in a swinging bucket rotor. *Methods Enzymol* **128**: 171–181, 1986.
12. Schafer JA and Barfuss DW, Mechanisms of transmembrane transport in isolated cells and their experimental study. In: *Membrane Transport of Antineoplastic Agents* (Ed. Goldman ID), pp. 1–37. Pergamon Press, Oxford, 1986.
13. Kötting J, Marschner NW, Neumüller W, Unger C and Eibl H, Hexadecylphosphocholine and octadecyl-methyl-glycero-3-phosphocholine: A comparison of hemolytic activity, serum binding and tissue distribution. In: *Alkylphosphocholines: New Drugs in Cancer Therapy* (Eds. Eibl H, Hilgard P and Unger C), Progress in Experimental Tumor Research, Vol. 34, pp. 131–142. Karger, Basel, 1992.
14. Portman OW and Illingworth DR, Lysolecithin binding to human and squirrel monkey plasma and tissue components. *Biochim Biophys Acta* **326**: 34–42, 1973.
15. Petersen ES, Kelley EE, Modest EJ and Burns CP, Membrane lipid modification and sensitivity of leukemic cells to the thioether lipid analogue BM 41.440. *Cancer Res* **52**: 6263–6269, 1992.
16. Stoll LL, Oskarsson HJ and Spector AA, Interaction of lysophosphatidylcholine with aortic endothelial cells. *Am J Physiol* **262**: H1853–H1860, 1992.
17. Spector AA, Plasma albumin as a lipoprotein. In: *Biochemistry and Biology of Plasma Lipoproteins* (Eds. Scanu AM and Spector AA), pp. 247–279. Marcel Dekker, New York, 1986.
18. Schnitzer JE, Carley WW and Palade GE, Specific albumin binding to microvascular endothelium in culture. *Am J Physiol* **254**: H425–H437, 1988.
19. Ockner RK, Weisiger RA and Gollan JL, Hepatic uptake of albumin-bound substances: Albumin receptor concept. *Am J Physiol* **245**: G13–G18, 1983.
20. Oram JF, Johnson CJ and Brown TA, Interaction of high density lipoprotein with its receptor on cultured fibroblasts and macrophages. Evidence for reversible binding at the cell surface without internalization. *J Biol Chem* **262**: 2405–2410, 1987.