Toxicity and disposition of TLC ELL-12 (liposomal antitumor ether lipid) in Sprague-Dawley rats

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TLC ELL-12 is a liposomal formulation of the antineoplastic L-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine [L-ET-18-OCH_3 (EL)]. The purpose of these studies was to characterize the toxicity and disposition of [*N*-methyl-^{14}C] L-ET-18-OCH_3 administered as TLC ELL-12. Rats received TLC ELL-12 by i.v. infusion into a tail vein as a single 12.5 or 62.5 mg/kg dose or as five daily doses at 12.5 mg/kg (cumulative dose of 62.5 mg/kg). Whole blood and tissue samples were collected over 24 h, and assayed for total and EL-specific radioactivity. The amounts of radioactivity in urine, bile, injection site and carcass were determined for up to 48 h. TLC ELL-12 was well tolerated in male and female rats in single doses up to 37.5 mg/kg. The minimum lethal dose was 112.5 mg/kg. Doses of 12.5 mg/kg (no observed adverse effects) and 62.5 mg/kg (approximate maximum tolerated dose) were chosen for further study. The pharmacokinetics of EL given as TLC ELL-12 were non-linear with a disproportionate increase in AUC at the higher dose. Daily dosing at 12.5 mg/kg did not result in accumulation in the blood. The highest concentrations of EL at 24 h after dosing were in the spleen and liver. Virtually no radioactivity was recovered in the urine or bile of rats, most remaining in the carcass and injection site (tail). After a 12.5 mg/kg dose of TLC ELL-12, the levels of EL in the blood and most tissues examined were well above the levels that inhibit tumor growth *in vitro* and may therefore be therapeutically active. *Anti-Cancer Drugs* 14:183–191

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Introduction

TLC ELL-12 is a liposomal formulation of the ether lipid, L-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (the L isomer of ET-18-OCH_3, L-ET-18-OCH_3). The racemic form of ET-18-OCH_3 was initially synthesized as a metabolically stable analog of the pharmacologically active but short-lived 2-lysophosphatidylcholine [1]. *rac*-ET-18-OCH_3 was found to be selectively toxic to cancer cells and has been investigated clinically as 'edelfosine' [2–5]. Effective use of *rac*-ET-18-OCH_3 has been limited by hemolytic, gastrointestinal and other toxicities, whether administered i.v. or orally.

When L-ET-18-OCH_3 is stably associated with complementary-shaped lipids, its hemolytic activity can be prevented [6,7]. The liposomal formulation found to be optimal in terms of toxicity and efficacy in murine models (TLC ELL-12) is composed of dioleoylphosphatidylcholine (DOPC):cholesterol (Chol):dioleoyl-phosphatidylethanolamine-derivatized glutaric acid (DOPE-GA):L-ET-18-OCH_3 in a 4:3:1:2 molar ratio [8].

TLC ELL-12 has been shown to inhibit the growth of a number of human and murine tumor cell lines *in vitro* with 50% growth-inhibitory concentrations in the range of 7.5–30 μM ($\sim$4–16 μg/ml) [9]. Intravenous treatment with TLC ELL-12 reduces the number of lung metastases in mice injected with Lewis lung carcinoma and B16/F10 melanoma at doses that are well tolerated [10].

The purpose of the current study was to determine the toxicity and disposition of L-ET-18-OCH_3 administered as TLC ELL-12 in normal, Sprague-Dawley rats after both single and multiple doses.

Potential metabolites of L-ET-18-OCH_3 include L-*O*-octadecyl-*sn*-glycero-3-phosphocholine (Lyso-EL), L-*O*-octadecyl-2-*O*-methyl-*sn*-glycerol-3-phosphate (PA) and L-*O*-octadecyl-2-*O*-methyl-*sn*-glycerol (GLY) (Fig. 1). To determine if any metabolites might contribute to the activity or toxicity of TLC ELL-12, the potential metabolites were either purchased or prepared in our laboratory, and their *in vitro* cytotoxicity and hemolytic activity were evaluated. TLC ELL-12, prepared with L-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-[*N*-methyl-^{14}C]-phosphocholine was used for pharmacokinetic studies. A high-performance liquid chromatography (HPLC)/radiochemical method was developed that was capable of resolving L-ET-18-OCH_3, Lyso–EL, PA and GLY from the

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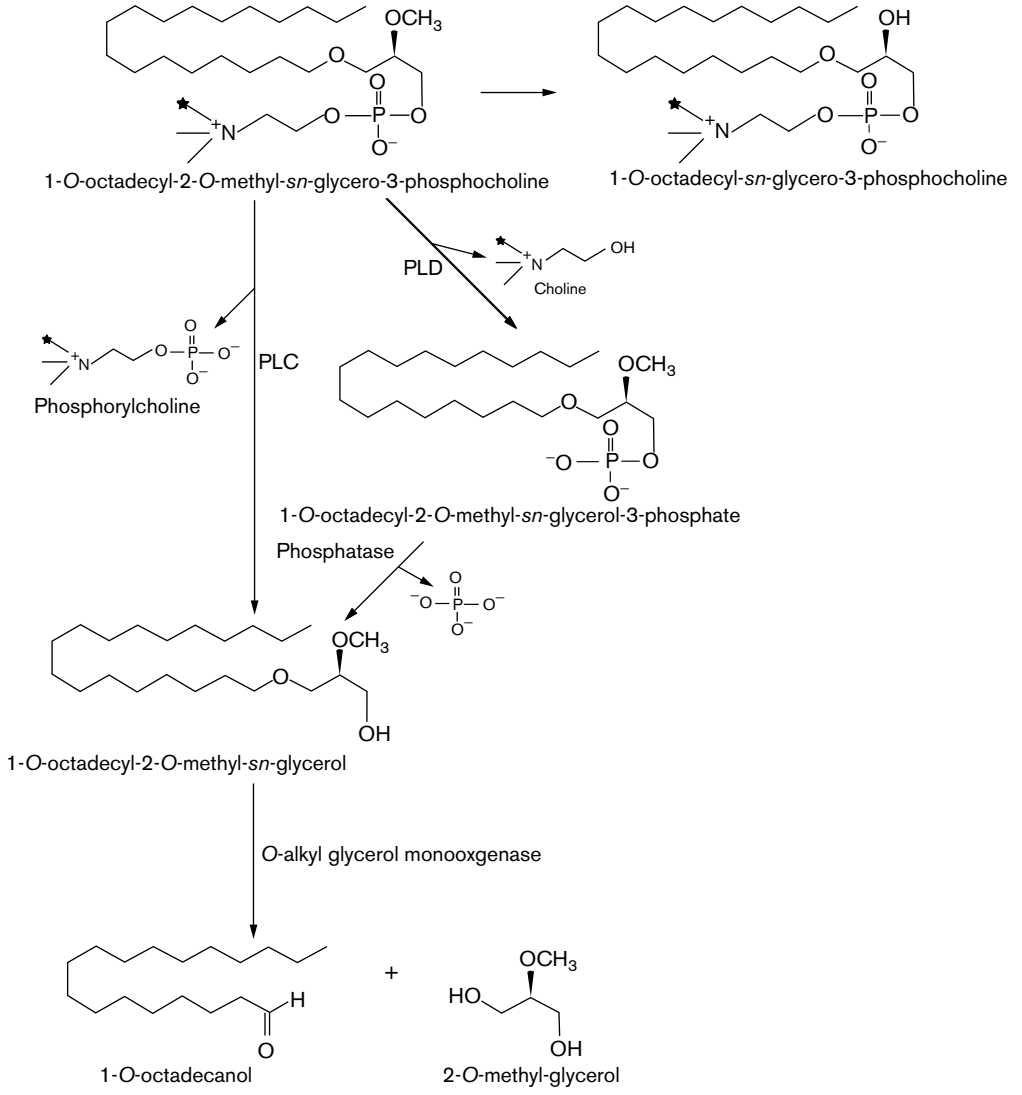
Fig. 1



Potential metabolic fate of L-ET-18-OCH_3 in rats. The asterisk indicates the position of the ^{14}C label used in the present studies.

more polar products (e.g. choline, phosphorylcholine, CDP-choline) and from the less polar products (e.g. the phospholipids as a class) that were likely to become labeled as a result of secondary metabolism. We then used this assay to determine the pharmacokinetics, tissue distribution and excretion of L-ET-18-OCH_3, and labeled metabolites after administration of TLC ELL-12.

Methods

Drug and chemicals

The L isomer of ET-18-OCH_3 was purchased from Avanti Polar Lipids (Alabaster, AL) and Alexis (San Diego, CA). The other lipids for liposome formulation were purchased from Avanti Polar Lipids. PA for use as an analytical standard was prepared by enzymatic hydrolysis of L-ET-18-OCH_3 using phospholipase D (from *Streptomyces chromofuscus*, type IV; Sigma, St Louis, MO) shown to be free of alkaline phosphatase activity. GLY for use as a standard was prepared by chemical reductive hydrolysis using $LiAlH_3$. The structures of the final products were confirmed by elemental analysis, nuclear magnetic resonance, mass and infrared spectroscopy. Both PA and GLY were estimated to be more than 98% pure by thin-layer chromatography and HPLC. Lyso-PAF (C_{18}) was purchased from Cayman Chemical (Ann Arbor, MI). Choline chloride, phosphocholine and phospholipids were purchased from Sigma. All lipids were stored desiccated at –20°C. Reagents for HPLC and scintillation counting were of analytical grade and were purchased from commercial suppliers.

TLC ELL-12 liposomes were prepared using a solvent evaporation method as previously described [7]. The final liposomal formulation contained 10 mg L-ET-18-OCH_3/ml in phosphate-buffered saline (PBS), pH 6.2. The mean diameter of the liposomes was ~100 nm as determined using a Nicomp model 370 submicron particle sizer system (Santa Barbara, CA). Final lipid composition of TLC ELL-12 was determined by HPLC using an evaporative light scattering detector. TLC ELL-12 was stored at 5 ± 3°C. All doses of TLC ELL-12 described here refer to the L-ET-18-OCH_3 content delivered in the TLC ELL-12 injections. For example, a dose of 12.5 mg/kg TLC ELL-12 refers to 12.5 mg/kg of L-ET-18-OCH_3 delivered as TLC ELL-12.

Radiolabeled TLC ELL-12 was prepared with L-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-[*N*-methyl-^{14}C]phosphocholine (^{14}C-L-ET-18-OCH_3) which had been synthesized from a reaction between ^{14}C-methyl-iodide with demethylated L-ET-18-OCH_3. The labeled liposomes were mixed with unlabeled liposomes to achieve the desired ^{14}C dose concentration.

In vitro studies

The cell lines and *in vitro* sulforhodamine B growth inhibition assay have been described in detail previously [9]. The GI_{50} values for each experiment were calculated using data obtained from three duplicate wells on two separate plates. The mean GI_{50} values and standard deviations were then calculated from the GI_{50}s from each independent experiment. For the *in vitro* hemolysis assay, fresh whole blood was collected from anesthetized (CO_2) Sprague-Dawley rats into EDTA monovets. A 4% red blood cell (RBC) solution was prepared by centrifuging the blood at 1000 r.p.m. to remove plasma and washing with Dulbecco's PBS (without Ca^{2+} and Mg^{2+}). Each compound tested was dissolved/dispersed in PBS to 2 times the final desired concentration. Equal volumes of the drug and RBC solution were mixed in glass test tubes, parafilmed and placed in a 37°C shaking incubator for 20 h at a speed of 140 r.p.m. All samples were run in duplicate. After incubation, the samples were centrifuged to remove any remaining intact RBCs. The supernatants were transferred to clean test tubes and measured for absorbance at 550 nm against a distilled water blank. The percent hemolysis was calculated by the following equation:(absorbance of supernatant (550 nm)/absorbance of sample completely lysed) × 100.

The complete hemolysis control was accomplished by repeated freeze–thaw cycles until the resulting pellet was absent or white. To better compare the hemolytic capabilities of the drugs, all drug concentrations were converted to μM. To account for interday variability of the 4% RBC solution, results were normalized to 0.4 OD. The concentration causing 50% hemolysis (HI_{50}) was calculated using WinNonlin version 1.1 model 106 (sigmoid Emax).

Animals and drug administration

Female and male Sprague-Dawley rats were purchased from Charles River (Raleigh, NC), and acclimated for at least 6 days prior to treatment or surgery. Animals were housed in individual cages, maintained on a 12 h light/12 h dark cycle, and given access to food and water *ad libitum*. All protocols and procedures were approved by our Institutional Animal Care and Use Committee.

For the single-dose toxicity study, TLC ELL-12 or saline was administered as an i.v. infusion over 20 min into the lateral tail vein of restrained rats (125–150 g at study initiation) using a butterfly infusion set connected to a Harvard infusion pump. TLC ELL-12 was diluted with 0.9% saline to achieve the appropriate doses in a volume of ~15 ml/kg. There were five rats per sex per dose level. After the infusion, the animals were returned to their cages and observed for 28 days. Animals were weighed daily. On day 28, samples of blood were collected and shipped on ice overnight to VETPATH (Tulsa, OK) for chemistry (glucose, blood urea nitrogen, calcium, phosphorous, total protein, albumin, globulin, cholesterol, total bilirubin, alkaline phosphatase, ALT, LDH, CPK, GGT, creatinine, amylase, sodium, potassium and chloride) and hematology (hemoglobin, hematocrit, red blood cell count, white blood cell count and platelet count).

For pharmacokinetic and tissue distribution studies, normal, female Sprague-Dawley rats (242–310 g) were surgically implanted with a jugular catheter for blood sample collection several days prior to drug administration and then assigned to one of three treatment regimens. In all of these regimens, restrained rats were given TLC ELL–12 containing ^{14}C-L-ET-18-OCH_3 via infusion into the tail vein using a constant L-ET-18-OCH_3 infusion rate of 25 mg/kg h using the infusion pump and butterfly infusion set. Each rat received approximately 56 μCi/kg/dose. One group of rats (Group A, $N = 6$) was given a single dose of TLC ELL–12 at 12.5 mg/kg, a second group (Group B, $N = 5$) received a single dose of 62.5 mg/kg and a third group (Group C, $N = 5$) received a daily dose of 12.5 mg/kg for 5 consecutive days (62.5 mg/kg cumulative dose). For the single-dose study (12.5 or 62.5 mg/kg), serial blood samples were obtained mid-infusion and at 0.083 (peak), 0.25, 0.5, 1, 2, 4, 8, 16 and 24 h post end infusion (PEI). For the multiple dose study, blood samples were collected at 5 min prior to start of infusion (trough) for days 2–5 and at 5 min PEI (peak) for days 1–5. Serial samples were obtained on day 5 at 0.25, 0.5, 1, 2, 4, 8, 16 and 24 h PEI. Aliquots of blood samples were transferred to glass screw-top scintillation vials and frozen at −20°C for subsequent processing for total radioactivity. The remaining blood

was transferred into polypropylene cryovials (Nalgene) and frozen at −70°C until further analysis. All animals were anesthetized with pentobarbital (i.v., 60–90 mg/kg) and exsanguinated by cardiac puncture 24 h after the start of their last infusion. Digestive organs were flushed with saline. Selected tissues were collected, weighed and stored frozen (−70°C) prior to homogenization and analysis. Tissues were homogenized in 50 ml polypropylene tubes with a Kinematica homogenizer with power unit (Brinkman Instrument, Westbury, NY) or hand-held homogenizers (Tissue Tearor; Biospec Products, Bartlesville, OK). Lung, spleen, stomach and muscle were homogenized after addition of 0.5 ml water (HPLC grade). Other organs were homogenized neat. Frozen carcasses were cut into small pieces and homogenized in a Waring blender after the addition of an aliquot of water (HPLC grade) equivalent to 40% of the carcass weight. Total and L-ET-18-OCH_3-specific radioactivity concentrations were determined as described below.

The excretion of L-ET-18-OCH_3 was studied in female, Sprague-Dawley rats given either a single 12.5 (Group D, $N = 4$) or 62.5 (Group E, $N = 4$) mg L-ET-18-OCH_3/kg dose as TLC ELL–12 by i.v. push injection. One day prior to dosing, animals were surgically implanted with a bile duct cannula and a bladder catheter under pentobarbital anesthesia. Bile and urine were collected in 1-h intervals from 0 to 4 h, in 2-h intervals from 4 to 8 h and in 8-h intervals from 8 to 48 h post-treatment. Animals were sacrificed at 48 h post-dosing. Total and L-ET-18-OCH_3-specific radioactivity were determined in urine, bile, injection site (tail) and carcass.

Analytical methods

Total radioactivity was determined by liquid scintillation counting (Beckman model LS580L LSC). L-ET-18-OCH_3-specific concentrations were determined using an HPLC method with radiochemical detection. One part blood, bile or tissue homogenate was extracted with 4, 3 or 9 parts isopropanol:water (80:20), respectively, and clarified by centrifugation. Recovery of L-ET-18-OCH_3 in the supernatant after extraction by this method was more than 90% for blank samples spiked with free L-ET-18-OCH_3 or TLC ELL-12. Samples (100 μl) were injected onto a Luna C_{18} column (5 μm, 4.6 × 150 mm; Phenomenex, Torrance, CA) equipped with a Luna C_{18} 5-μm guard column and maintained at a temperature of 45°C. Separation was accomplished by gradient elution at a flow rate of 1.25 ml/min using a Waters 626 pump and 600S controller as a solvent delivery system. Total run time was 40 min.The column was held at 100% A for the first 15 min where solvent A is methanol:water:trifluoroacetic acid (90:10:0.1). Over the next 5 min the solvent composition ratio changed from 100% A to 100% B, which is methanol:isopropanol:water:trifluoroacetic acid (20:75:5:0.1), and was maintained for another 15 min before changing back to solvent A (100%) over a 2-min interval. The column was allowed to reach equilibrium for 5 min before analysis of the next sample. Detection of radioactive compounds was accomplished with a radiochemical detector using external quench correction (radiomatic flow scintillation analyzer model 500TR series; Packard, Meriden, CT). Detection of non-isotopes was achieved using an evaporative light scattering detector (model 500; Alltech, Deerfield, IL), and was used to ensure that resolution was achieved for L-ET-18-OCH_3 and potential metabolites. Performance of the HPLC column and radiochemical detector was evaluated on each study day by preparation of standards in whole blood, plasma and tissue homogenate containing ^{14}C-L-ET-18-OCH_3 in the range of 10 000–5 000 000 d.p.m./ml or d.p.m./g. The radiolabel efficiency curve for the radiochemical detector was used to obtain readbacks for the samples. The lower limit of measurement was set at approximately 400 d.p.m./peak for each tissue type with relative standard deviations of 14.5–29.7%. Based on the specific activity of 4.40 μCi/mg of ^{14}C-L-ET-18-OCH_3, the lower limit of quantitation for blood, bile and tissue homogenate were estimated at 2.0 μg/ml, 1.6 μg/ml and 4.0 μg/g, respectively.

L-ET-18-OCH_3 and its potential metabolites, Lyso-EL, PA and GLY, were resolved. More polar compounds which were expected to become labeled as a consequence of secondary metabolism *in vivo* (choline, phosphorylcholine and CDP-choline) eluted as a single peak (peak 1) at or near the solvent front, while the less polar compounds (e.g. phospholipids) eluted as a single peak (peak 2) after GLY (Fig. 2).

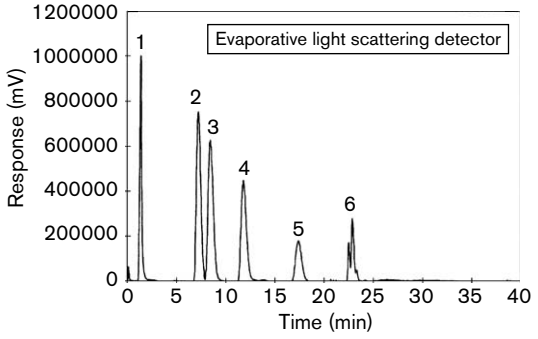
Pharmacokinetic parameters for EL in whole blood were estimated using non-compartmental methods (WinNonlin version 1.5; Pharsight, Mountain View, CA).

Results

In vitro cytotoxicity and hemolytic activity

The potential metabolites of L-ET-18-OCH_3 (Lyso-EL, PA, GLY, stearic acid and stearyl alcohol) were tested for their growth-inhibitory and hemolytic activities, and compared with L-ET-18-OCH_3. The glycerol metabolite (GLY) showed the greatest activity with GI_{50}s ranging from about 8.9 to 31.6 μM, but in all cases was less potent than L-ET-18-OCH_3. The other potential metabolites were essentially inactive ($GI_{50} > 100$ μM) or showed some activity, but much less than L-ET-18-OCH_3 (Table 1). As expected, the lyso metabolite showed hemolytic activity comparable to L-ET-18-OCH_3 ($H_{50} = 18.34$ μM for L-ET-18-OCH_3 and 18.66 μM for Lyso-EL). PA showed some hemolytic activity at concentrations about 10-fold higher than L-ET-18-OCH_3 ($H_{50} = 176.55$ μM). The other metabolites had little or no hemolytic activity (Fig. 3).

Fig. 2



Peak	RT	Compound (s)	Abbreviation	^{14}C-?
1	1.5	phosphorylcholine, choline, cytidine-diphosphocholine		Y
2	6.5	1-*O*-octadecyl-*sn*-glycero-3-phosphocholine	lyso-EL	Y
3	8.7	1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine	EL	Y
4	12.5	1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphate	PA	N
5	17	1-*O*-octadecyl-2-*O*-methyl-*sn*-glycerol	GLY	N
6	23.5	phosphatidylcholine, phosphatidylethanolamine	PC, PE	Y

Chromatographic separation of EL from its potential metabolites.

Table 1 ***In vitro*** **growth inhibition of tumor cell lines after 72 h treatment [GI_{50} (μM ± SD)]**

Compound	A549	Lewis lung	MCF7	MCF7/adr	L1210	L1210/vmdr	U937
L-ET-18-OCH_3	9.1 ± 0.4	29.1 ± 2.1	18.6 ± 4.6	27.9 ± 2.1	4.8 ± 0.4	8.8 ± 1.5	1.5 ± 0.4
GLY	28.1 ± 1.4	25.9 ± 1.6	31.6 ± 1.2	31.5 ± 0.8	30.8 ± 2.4	29.4 ± 2.9	8.9 ± 0.5
PA	>100	>100	>100	>100	>100	>100	40.8 ± 1.8[b]
Lyso-EL	>90.6 ± 0.0[a]	33.6 ± 5.3	>100	40.9 ± 5.6	28.7 ± 4.6	26.8 ± 3.8	27.9 ± 3.8
Octadecanol	>93.2[a]	52.6 ± 2.5	>54.5 ± 10.0[a]	>100	>100	>100	42.5 ± 1.5
Stearic acid	31.0 ± 0.2	41.9 ± 3.6	>100	37.6 ± 3.7	>100	>72.9 ± 23.9[a]	47.4 ± 3.6

[a]At least one GI_{50} value in the group (of three) was >100 μM.
[b]The vehicle (1.8% ethanol at the highest test concentration) may have contributed to an inhibition in cell growth.

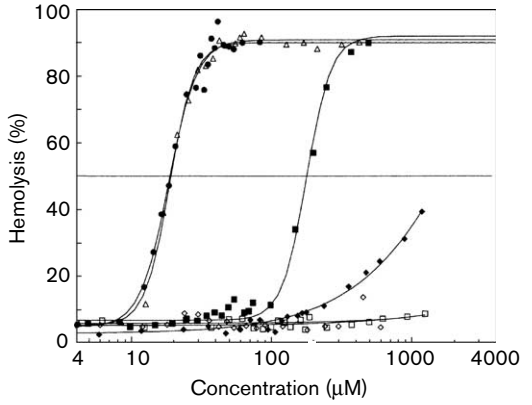
Toxicity

The initial toxicology studies were conducted to determine appropriate doses for subsequent studies; therefore, the range of doses was large. The minimum lethal single dose of TLC ELL-12 in rats was 112.5 mg/kg (Table 2). All deaths in the 150 mg/kg dose group as well as the single death in the 112.5 mg/kg dose group occurred within 3–24 h after dosing. Clinical signs in the two high-dose groups (112.5 and 150 mg/kg) were lethargy, tremors, diarrhea, pink or blue ears and paws, and rapid breathing. These signs disappeared in all surviving animals within 1 day after dosing. Surviving animals in the two high-dose groups lost body weight on the day after dosing, but showed normal weight gains by day 3 post-dose. There was a slight decrease in body weight (~3–4%) in animals in the 37.5 mg/kg group. There were no clear treatment-related effects on hematology or serum chemistry at 28 days post-treatment in any group. The no observed adverse effect level was 12.5 mg/kg.

Pharmacokinetics of EL in whole blood

Mean whole blood concentration–time profiles for the single dose groups (Group A, 12.5 mg/kg and Group B, 62.5 mg/kg) are compared in Figure 4. In both cases the decline of L-ET-18-OCH_3 appeared to be multiphasic. Dose dependency over this dose range is suggested by an apparent decrease in the rate of decline at the higher dose level. Mean whole blood concentration–time profiles

Fig. 3



Hemolysis of washed rat RBCs by EL and its potential metabolites: filled circles, EL; open triangles, lyso-EL; filled squares, PA; open diamonds, glycerol; open squares, S-OH; filled diamonds. S-acid.

Table 2 Single i.v. dose study of TLC ELL-12 in Sprague-Dawley rats

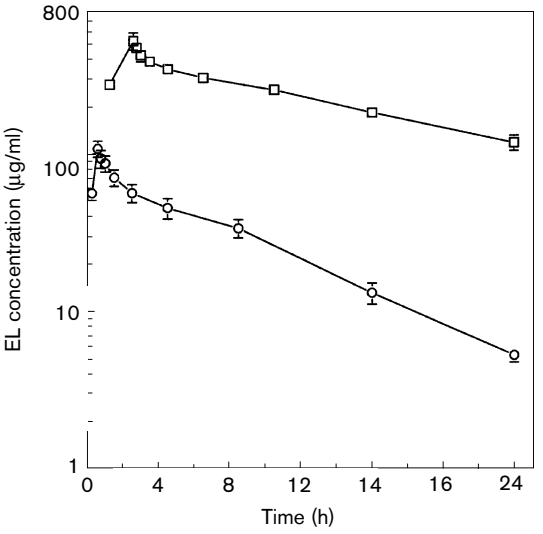
Group	Rx	L-ET-18-OCH_3 dose (mg/kg)	Total lipid dose (mg/kg)	No. dead/total no. treated		Day of death (no. animals)	
				M	F	M	F
1	saline	0	0	0/5	0/5		
2	ELL-12	12.5	75	0/5	0/5		
3	ELL-12	37.5	225	0/5	0/5		
4	ELL-12	112.5	675	1/5	0/5	1 (1)	
5	ELL-12	150	900	1/5	3/5	1 (1)	1 (3)

for the dose groups receiving 12.5 mg/kg either as a single dose (Group A) or as five daily doses (Group C) are compared in Figure 5. Examination of the profiles and the trough values suggest only a modest accumulation of L-ET-18-OCH_3 with repeated dosing on this schedule.

Table 3 shows the mean pharmacokinetic parameters for L-ET-18-OCH_3 in whole blood of rats in Groups A, B and C. There was a 10-fold increase in AUC and a decrease in clearance in rats receiving 62.5 mg/kg (Group B) compared with those receiving 12.5 mg/kg (Group A), suggesting dose-dependent pharmacokinetics in this dose range. The AUC and clearance of L-ET-18-OCH_3 in rats after five daily doses of 12.5 mg/kg TLC ELL-12 (Group C) was not different from that after a single dose (Group A).

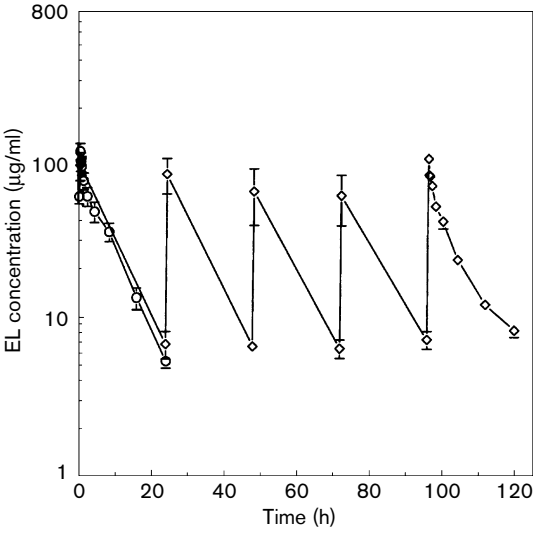
Most (more than 90%) of the circulating radioactivity in rats given single doses of TLC ELL-12 was in the form of the parent drug (L-ET-18-OCH_3) up to 16 h after the end of the infusion. At 24 h after the end of the infusion, up to about 20% of the blood radioactivity in Group A rats was found in peak 1 (choline, phosphorylcholine) or peak 2 (phospholipids) as shown in Figure 6. A greater amount of radioactivity (up to about 40%) was present in the blood

Fig. 4



Comparison of mean (± SEM.) whole blood concentration–time profiles of EL for rats receiving a single 12.5 mg/kg (circles) or 62.5 mg/kg (squares) i.v. infusion (25 mg EL/kg/h) of TLC ELL-12. There were five or six rats in each group.

Fig. 5



Comparison of mean (± SEM) whole blood concentration time profiles of EL for rats receiving a single (circles) or five daily (diamonds) 12.5 mg/kg doses of TLC ELL-12. There were five or six rats in each group.

in non-L-ET-18-OCH_3 peaks after five daily doses of TLC ELL-12 (Group C, Fig. 6). Lyso-EL was not detected in the blood of any rat.

Tissue distribution of L-ET-18-OCH_3 and metabolites

Mean tissue concentrations of L-ET-18-OCH_3 in selected organs at 24 h after the last dose are shown in Figure 6.

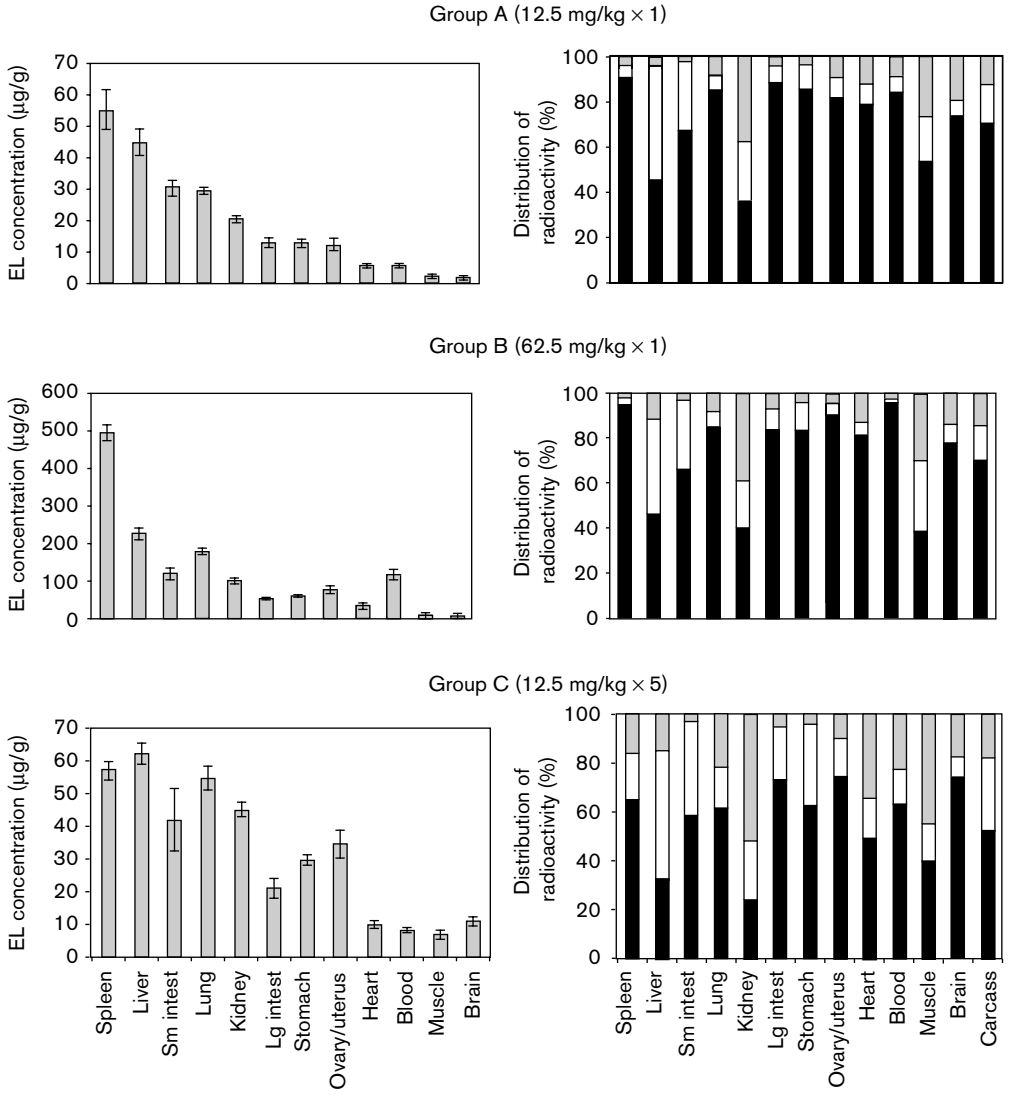
Table 3 Mean ± SD pharmacokinetic parameters for L-ET-18-OCH_3 in blood of rats after the last infusion of TLC ELL-12

	Group A (12.5 mg/kg × 1)	Group B (62.5 mg/kg × 1)	Group C (12.5 mg/kg × 5)
N	6	5	5
C_{max} (μg/ml)	107.8 ± 31.0	521.7 ± 113.2	95.5 ± 11.0
$T_{1/2}$ (h)[a]	5.9 ± 2.0	12.1 ± 3.9	9.2 ± 2.6
$AUC_{0-\infty}$ (μg·h/ml)	734 ± 197	7879 ± 1967	699 ± 102
CL (l/kg·h)	0.018 ± 0.006	0.008 ± 0.002	0.018 ± 0.003
V_{ss} (l/kg)	0.171 ± 0.109	0.145 ± 0.013	0.217 ± 0.023

[a]Harmonic mean ± pseudo SD.

The highest concentrations of L-ET-18-OCH_3 were found in the spleen and liver, followed by organs of the digestive tract, lungs, kidneys and ovaries/uterus. Lower concentrations were found in heart, muscle and brain. The blood, necropsied organs and carcass accounted for 78–86% of the administered ^{14}C radioactivity in the single-dose treatment groups and for 59% in the group receiving five daily doses. Tissues with substantial radioactivity in the form of labeled endogenous compounds (peaks 1 and 2) were the liver, kidney, muscle and, to a lesser extent, the organs of the digestive tract (Figure 6). The distribution of radioactivity between L-ET-18-OCH_3 and its metabolites in the organs evaluated appeared to be similar in animals receiving single 12.5 and 62.5 mg/kg doses. Organs which may accumulate EL on repeated dosing are the liver, lung, kidney, stomach, ovary/uterus and brain.

Fig. 6



Tissue distribution of EL and products of secondary metabolism as determined by HPLC with radiometric detection in rats at 24 h after the end of the last infusion. The left panels show the mean (± SEM) EL concentrations in each tissue and the right panels show the mean distribution of total radioactivity among EL (solid) and peak 1 (choline, phosphorylcholine, CDP-choline; open) and peak 2 (phospholipids; half-tone).

Table 4 Mean ± SD cumulative total and L-ET-18-OCH_3-specific radioactivity (% dose) recovered 48 h after a single, bolus, i.v. injection of TLC ELL-12 in rats

	Group D (12.5 mg/kg)		Group E (62.5 mg/kg)	
	L-ET-18-OCH_3 recovered (% dose)	Total radioactivity (% dose)	L-ET-18-OCH_3 recovered (% dose)	Total radioactivity (% dose)
Tail	ND	9.6 ± 7.3	ND	11.1 ± 9.3
Urine	ND	0.413 ± 0.366	ND	0.776 ± 0.612
Bile	0.0023 ± 0.0013	2.030 ± 0.316	0.0002 ± 0.0001	1.198 ± 0.549
Carcass	45.8 ± 3.902	77.0 ± 7.0	63.2 ± 0.919	99.9 ± 2.5
Total	45.8 ± 3.902	89.1 ± 4.1	63.2 ± 0.919	113.3 ± 10.7

ND = not done.

Excretion

Table 4 shows that virtually no radioactivity was recovered in the urine or bile of female rats within 48 h of a single dose of 12.5. or 62.5 mg L-ET-18-OCH_3/kg as TLC ELL-12 containing ^{14}C-L-ET-18-OCH_3 by i.v. push injection. Most radioactivity was accounted for in the carcass and injection site (tail). In the carcass, more than 89% of the total radioactivity was recovered with 45–64% of the L-ET-18-OCH_3 dose present unchanged.

Discussion

Biologically active, metabolically stable ether lipids offer a novel approach to the treatment of neoplastic disease. Unlike most currently available cytotoxic drugs, ether lipids do not appear to interact with DNA. The mechanism(s) of tumor cell growth inhibition and cytotoxicity of L-ET-18-OCH_3 are not well understood, but may involve immunological modulation [12] as well as direct effects on signal transduction [13]. The basis for the selective effect on tumor cells is also presently unknown. It has been suggested that the selectivity is due to differential uptake or metabolism in tumor versus normal cells [14].

Although racemic ET-18-OCH_3 was synthesized over 30 years ago and has shown some therapeutic activity against non-small cell lung cancer in limited human trials, hemolytic and gastrointestinal toxicity have limited its usefulness. Incorporation of L-ET-18-OCH_3 into stable liposomes (TLC ELL-12) has allowed i.v. dosing without these toxicities in animals and a phase I trial in patients with advanced solid tumors is in progress [15].

The purpose of the current studies was to evaluate the toxicity and disposition of L-ET-18-OCH_3 when administered i.v. to rats as TLC ELL-12. The initial toxicity study in rats showed that TLC ELL-12 given by i.v. infusion was well tolerated in single-doses up to 37.5 mg/kg with only slight (3–4%) weight loss for several days after dosing the only observed effect. The minimal lethal dose was 112.5 mg/kg with all deaths occurring within 24 h of dosing. The no observed effect level was 12.5 mg/kg. No changes in serum chemistry or hematology were detected in surviving animals 28 days after dosing.

Two dose levels were chosen for the pharmacokinetic studies—12.5 mg/kg, which is a non-toxic but potentially therapeutic dose (based on studies in murine tumor models [10]), and 62.5 mg/kg, which would be in the range of the maximum non-lethal dose.

We found that the pharmacokinetics of L-ET-18-OCH_3 after administration of TLC ELL-12 was non-linear in rats in the dose range of 12.5–62.5 mg/kg with a disproportionate increase in the AUC at the higher dose. Daily dosing at 12.5 mg/kg did not result in accumulation in the blood. Radioactivity found in most tissues 24 h after a single dose was in the form of the parent drug, with the exception of the liver, kidney and muscle, where about half of the radioactivity was found as products of secondary metabolism. L-ET-18-OCH_3 was initially synthesized as an analog of 2-lysophosphocholine that would not be rapidly inactivated by lysophospholipases and acyltransferases. Magistrelli *et al.* [14], using racemic ET-18-OCH_3 labeled with ^{3}H in position 9–10 of the 1-alkyl chain and thin-layer chromatography for separation, showed that 98% of EL remained unchanged after 24 h incubation with HL60, K562 and HT29 tumor cell lines. Similarly, plasma and erythrocytes from rats metabolized only 4–5% of the original compound in 3 h. However, in cultured hepatocytes, 58.3% of the label was present at 24 h as the metabolites GLY, PA and stearyl alcohol (products of direct hydrolysis by phospholipases C and D), and phospholipids and neutral lipids (products of secondary metabolism). Our results with L-ET-18-OCH_3 in TLC ELL-12 in rats *in vivo* are consistent with these findings.

Like other agents that act by inhibiting specific signal transduction events [16], the therapeutic efficacy of ET-18-OCH_3 is related more to the duration of treatment than dose [11]. Thus, maintaining effective levels of L-ET-18-OCH_3 in tumor tissue may be more important than achieving high plasma or tissue levels. These studies suggest that the pharmacokinetics of L-ET-18-OCH_3 delivered in TLC ELL-12 are dominated by distributional clearance (i.e. uptake into tissues). L-ET-18-OCH_3 in the tissues then undergoes slow metabolism and re-circulation into the endogenous lipid pool, probably by

the same enzymes that metabolize natural lipids. The persistence of the L-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine in tissues suggests that relevant levels, once attained, could theoretically be maintained with relatively infrequent maintenance dosing.

In conclusion, after a 12.5 mg/kg dose of TLC ELL-12 (a dose level that is well-tolerated), the levels of L-ET-18-OCH_3 in the blood and most tissues examined were well above the levels that inhibit tumor growth *in vitro*, and are therefore likely to be therapeutically active.

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