Activity, pharmacokinetics and tissue distribution of TLC ELL-12 (liposomal antitumor ether lipid) in rats with transplantable, s.c. methylnitrosourea-induced tumors

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TLC ELL-12 is a liposomal formulation of the novel antineoplastic compound 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (L-ET-18-OCH_3). The purpose of these studies was to evaluate the activity and tissue distribution of L-ET-18-OCH_3 when administered i.v. as TLC ELL-12 to rats bearing solid tumors. Growth-inhibitory activity of L-ET-18-OCH_3 and TLC ELL-12 against methylnitrosourea (MNU)-induced tumors grown *in vitro* was evaluated. Female Buffalo rats were injected s.c. with transplantable MNU-induced tumor cells. Four days later, animals were treated i.v. with L-ET-18-OCH_3 administered as TLC ELL-12 once daily for 5 consecutive days. Another group of MNU-tumor bearing rats was given a single 12.5 mg/kg dose of TLC ELL-12 containing [^{14}C]L-ET-18-OCH_3 by i.v. injection into a tail vein. The 50% growth inhibitory concentration for TLC ELL-12 against MNU tumor cells *in vitro* was 63 μM (about 30 μg/ml). Tumor growth was significantly inhibited in ELL-12-treated rats versus controls. After a single dose, whole blood L-ET-18-OCH_3 concentrations declined in a multiphasic fashion with C_{max} and terminal half-life values of approximately 91.1 μg L-ET-18-OCH_3/ml and 13.1 h, respectively. Tumor L-ET-18-OCH_3 levels increased through the first 16–24 h post-dosing to about 23 μg/g and remained elevated at the terminal time point with little evidence of metabolism. Concentration–time profiles for selected tissues indicate rapid distribution of L-ET-18-OCH_3 from the circulation into tissues with highest concentrations in spleen, liver, lungs, kidneys and gastrointestinal tract. L-ET-18-OCH_3 as TLC ELL-12 shows both *in vitro* and *in vivo* activity against the MNU tumor line. When i.v. administered, L-ET-18-OCH_3 from ELL-12 is well distributed and slowly eliminated by metabolism in tissues. *Anti-Cancer Drugs* 14:481–486 © 2003 Lippincott Williams & Wilkins.

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Introduction

TLC ELL-12 is a liposomal formulation of the ether lipid, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (the 'L' isomer of edelfosine or L-ET-18-OCH_3) designed to reduce the non-specific hemolytic toxicity of this antitumor agent [1,2]. 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 (about 4–16 μg/ml) [3]. 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 [4].

Previous studies utilizing TLC ELL-12, prepared with 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-[*N*-methyl-^{14}C]-phosphocholine and high-performance liquid chromatography (HPLC) with radiochemical detection demonstrated that levels of 10–100 μg/ml L-ET-18-OCH_3 in the blood and 10–50 μg/g in tissues (liver, spleen, kidney, lung and intestines) were achieved in normal, non-tumor-bearing rats, after single i.v. doses of 12.5 mg/kg [5]. These studies suggested that the pharmacokinetics of L-ET-18-OCH_3 delivered in TLC ELL-12 are dominated by distributional clearance (i.e. uptake into tissues) and that L-ET-18-OCH_3 then undergoes slow metabolism by the same enzymes that metabolize endogenous lipids. We were interested in determining the disposition of L-ET-18-OCH_3 in tumor tissue after doses that were likely to be therapeutically active. A methylnitrosourea (MNU)-induced tumor model was chosen as these tumors are transplantable, will grow reproducibly and homogeneously in rats, and have been reported to respond to systemic treatment with racemic ET-18-OCH_3 [6,7].

Methods

Drugs and chemicals

The L-isomer of ET-18-OCH_3 was purchased from Avanti Polar Lipids (Alabaster, AL) and Alexis (San Diego, CA).

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The other lipids for liposome formulation were purchased from Avanti Polar Lipids. Standards for the development of the bioanalytical assay were either prepared from L-ET-18-OCH_3 or purchased where commercially available as described previously [5]. 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 [2,3,5]. The final liposomal formulation contained 10 mg L-ET-18-OCH_3/ml in phosphate-buffered saline, pH 6.2. The mean diameter of the liposomes was around 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 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-[*N*-methyl-^{14}C]phosphocholine as described previously [5]. The labeled liposomes were mixed with unlabeled liposomes to achieve the desired ^{14}C dose concentration.

Cell line and *in vitro* studies

Adherent MNU-derived rat tumor cell line MNU/Buff Mammary Carcinoma was obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, DCTDC Tumor Repository, Frederick, MD). Cells were grown in Medium 199 (with Earle's salts, L-glutamine, sodium bicarbonate and without phenol red, containing 10% v/v fetal bovine serum and 25 μM gentamicin). Cells were incubated at 37°C, 100% humidity and 5% CO_2, and were dissociated with Trypsin-Versene for passaging and *in vitro* studies. The cells were passaged *in vivo* after washing with cold Medium 199 by s.c. injection in the ventral region of female Buffalo rats. Tumors (about 25×15 mm) were excised, sliced and pressed through a 40 mesh screen to form a single homogenous pool (100 mg tumor cells/ml Medium 199) for experimental implantation.

A sulforhodamine B (SRB) assay was employed to determine relative *in vitro* drug sensitivity using the GI_{50} parameter (the concentration of drug which inhibits cell proliferation by 50% versus controls). The detailed methodology of the SRB assay is described elsewhere [3]. The GI_{50} values were calculated using data obtained from three duplicate wells on two separate plates.

Animals and drug administration

Female Buffalo (130–150 g; about 25 days old) rats, originally from the National Institutes of Health, were purchased from Charles River Laboratories (Raleigh, NC) and acclimated for at least 6 days prior to tumor implantation. 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.

Tumor cells (20 mg in a volume of 0.2 ml per rat) were implanted s.c. in the ventral region of female Buffalo rats. For the efficacy experiment, animals with palpable tumors were randomized into three groups (six to nine rats per group) on day 4 after tumor implantation. Treatment was given by bolus i.v. injection via the tail vein for 5 consecutive days beginning on day 4. Every other day, the length (longest dimension) and width (shorter dimension) of the tumors were measured (in mm) with calipers. Tumor volume was calculated as follows: volume (in mm^3) = $[\text{length} \times (\text{width}/2)^2] \times \pi$.

For the pharmacokinetic experiment, 50 rats were initially implanted with tumors. On the day prior to treatment, tumors were measured and evaluated. Thirty-six animals were selected for use on the basis of the shape of the tumor mass, and its ease of dissection and tumor size relative to that of the population mean. TLC ELL-12 containing 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-[*N*-methyl-^{14}C]phosphocholine was administered by i.v. push injection into a lateral tail vein at a dose of 12.5 mg/kg. Animals bearing tumors but not treated were necropsied to obtain blank tissues. Groups of three to four rats were sacrificed at 0.25, 0.5, 1, 2, 4, 8, 16, 24 and 48 h post-dosing. Animals were anesthetized with pentobarbital (60–90 mg/kg i.v.) and exsanguinated by cardiac puncture using a 10-ml syringe with EDTA as anticoagulant. 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 samples were transferred into polypropylene cryovials (Nalgene; Nalge, Rochester, NY) and frozen at –70°C until further analysis. 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, Bartlesville, OK). Lung, spleen, stomach and muscle were homogenized after addition of 0.5 ml water (HPLC grade). Other organs were homogenized neat.

Total radioactivity was determined by liquid scintillation counting (model LS5801 LSC; Beckman, Fullerton, CA)

and L-ET-18-OCH_3-specific concentrations were determined using an HPLC method with radiochemical detection as previously described [5]. This chromatographic method was capable of resolving the parent drug from potential radioactive metabolites and from classes of endogenous compounds which were likely to become labeled as a consequence of that metabolism. The latter included polar (peak 1: choline, phosphorylcholine, cytidine-diphosphocholine) and non-polar (peak 2: phospholipids) intermediary metabolites. The lower limits of measurement was set at approximately 400 d.p.m./peak for each tissue type. Based on the specific activity of 10.85 μCi/mg of ^{14}C-L-ET-18-OCH_3, the lower limits of quantitation for blood and tissue homogenate samples were estimated at 0.8 μg/ml and 1.7 μg/g, respectively. Pharmacokinetic parameters for L-ET-18-OCH_3 were estimated using the mean concentration of the three to four rats at each time point and non-compartmental methods (Winnonlin version 1.5; Pharsight, Mountain View, CA).

Results

In vitro cytotoxicity

The mean (± SD) 50% growth inhibitory concentration of L-ET-18-OCH_3 against MNU/Buff tumor cells *in vitro* was 32.0 ± 1. 2 μmol/l (about 16.8 μg/ml). The mean GI_{50} for liposomal L-ET-18-OCH_3 (TLC ELL-12) was 62.9 ± 6.2 μmol/l (about 32.9 μg/ml).

In vivo efficacy

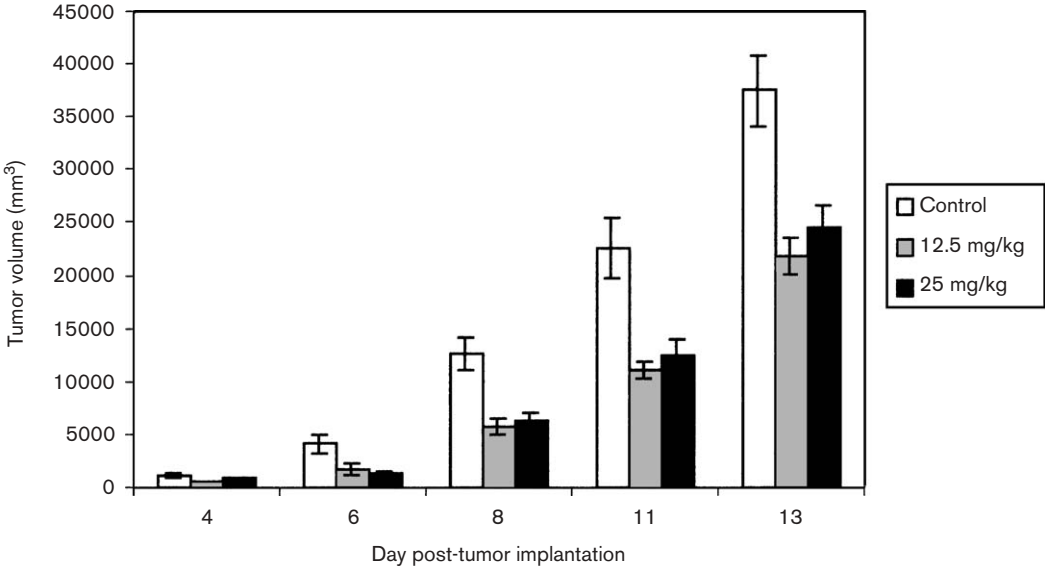
Figure 1 shows the mean tumor volume of rats implanted with the MNU/Buff tumor and treated with either saline (control) or TLC ELL-12. TLC ELL-12 delayed tumor growth at a dose of 12.5 mg/kg relative to the control animals. A higher dose of TLC ELL-12 (25 mg/kg) did not show any greater antitumor activity.

Pharmacokinetics and tissue distribution of L-ET-18-OCH_3

Female, Buffalo rats weighing 0.139 ± 0. 002 kg and with mean tumor sizes of 1193 ± 42 mm^3 (range 804–1780 mm^3) were treated with 12.5 mg/kg of TLC ELL-12 (undiluted) by slow bolus, i.v. injection. This dose corresponded to approximately 77 mg total lipid/kg. The dose volume was 1.30 ml/kg and the radioactivity was approximately 138 μCi/kg as 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-[*N*-methyl-^{14}C]phosphocholine.

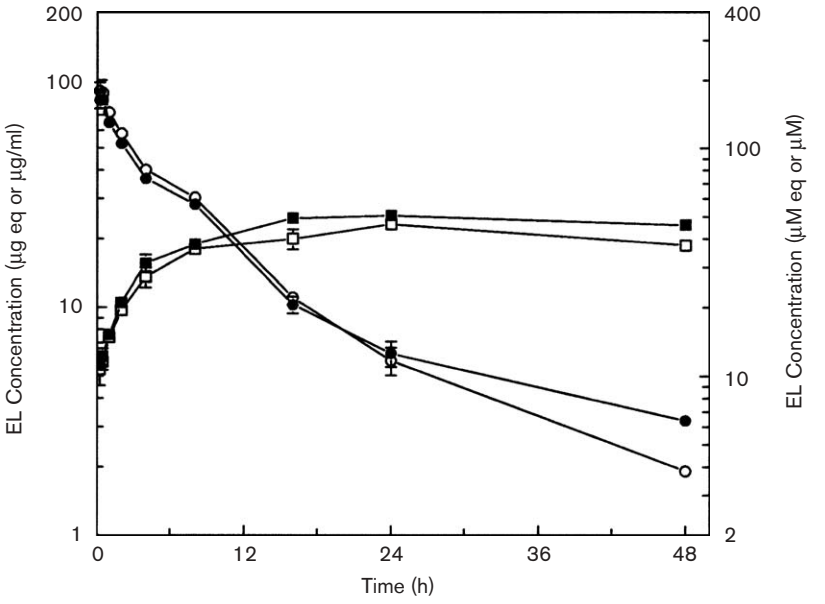
Concentration–time profiles comparing blood and tumor L-ET-18-OCH_3 levels (determined with a specific HPLC assay with radiochemical detection) with those estimated on the basis of total ^{14}C radioactivity are shown in Fig. 2. Whole blood L-ET-18-OCH_3 concentrations decline with time in a multiphasic fashion with C_{max} and terminal half-life values of approximately 91.1 μg/ml and 13.1 h, respectively. Clearance and V_{ss} were estimated at 0.016 l/h · kg and 0.203 l/kg, respectively. Tumor L-ET-18-OCH_3 levels increased through the first 16–24 h post-dosing and remained elevated at the terminal 48-h time point. Tumor levels of L-ET-10-OCH_3 after a single 12.5 mg/kg i.v. dose of TLC ELL-12 were in the range of the *in vitro* GI_{50} for this tumor and were higher than that noted for most other tumor cell lines [3]. Tumor profiles obtained using L-ET-18-OCH_3 concentrations calculated

Fig. 1



Mean tumor volume (± SEM) in rats implanted with MNU/Buff tumor cells and treated with saline (control) or TLC ELL-12 i.v. on days 4, 5, 6, 7 and 8 post-tumor implantation.

Fig. 2



Whole blood (circles) and tumor (squares) concentrations of L-ET-18-OCH_3 (EL) estimated by total radioactivity (filled symbols) and specific HPLC assay (open symbols) in rats after a single i.v. dose of TLC ELL-12 (12.5 mg/kg).

on the basis of total ^{14}C radioactivity are similar to those obtained using the specific L-ET-18-OCH_3 assay suggesting little evidence of metabolism.

Table 1 shows pharmacokinetic parameters for tissues analyzed by HPLC with radiochemical detection. These data suggest rapid distribution of drug from circulation into the tissues or rapid uptake of L-ET-18-OCH_3 as TLC ELL-12. Time taken to reach the highest concentration (T_{max}) ranged from 0.25 to 8 h for most tissues except for ovaries and uterus, where T_{max} was 24 h (Table 1). Tissue clearance of L-ET-18-OCH_3 was fairly rapid with terminal half-lifes ($T_{1/2}$) of most tissues in the range of 14–67 h (median 35 h). The longest $T_{1/2}$ of 197 h was observed for the tumor. Area under the curve (AUC_{0-48}) and C_{max} was highest for the spleen compared to the other organs studied.

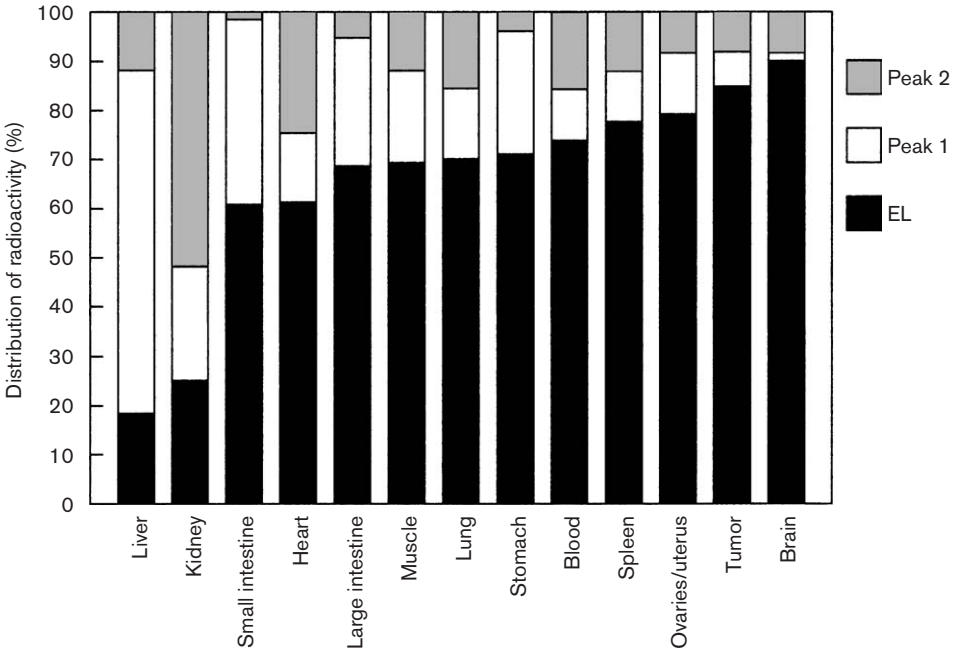
Mean tissue L-ET-18-OCH_3 concentrations calculated on the basis of total ^{14}C radioactivity were similar to those obtained using the specific L-ET-18-OCH_3 assay in most tissues, particularly at the earlier time points. The distribution of radioactivity (^{14}C) as percent of L-ET-18-OCH_3, peak 1 and peak 2 at 48 h post-dose are given in Fig. 3. There was a good agreement between the total ^{14}C radioactivity as determined by liquid scintillation

Table 1 Tissue pharmacokinetic parameters for L-ET-18-OCH_3 after i.v. administration of TLC ELL-12 (12.5 mg/kg)

Organ/fluid	C_{max} (µg/ml)	T_{max} (h)	$T_{1/2}$ (h)[a]	AUC_{0-48} (µg·h/ml)
Blood	91.1	0[b]	13.1	716
Tumor	23.2	24	197	928
Liver	54.0	4	15	1178
Spleen	153.9	8	14	4259
Kidney	38.5	0.25	32	883
Stomach	15.0	8	67	538
Small intestine	33.5	8	27	1144
Large intestine	17.7	8	27	625
Heart	24.9	0.25	46	271
Lung	53.8	0.25	35	1169
Brain	3.9	1.0	NC[c]	145
Muscle	6.3	0.25	35	124
Ovaries/uterus	11.4	24	37	458

[a]In instances where the calculated value is longer than the 48-h sampling period, the reported value is at best an estimate.
[b]Back extrapolated to $t = 0$.
[c]Not calculable.

Fig. 3



Mean percent distribution of total radioactivity eluting as L-ET-18-OCH_3 (EL), polar secondary metabolites (peak 1) or non-polar secondary metabolites (peak 2) in tissue samples collected at 48 h post-dose of TLC ELL-12 (12.5 mg/kg).

counting of tissue homogenates and the total radioactivity recovered on the HPLC column. Major sites for metabolism include the liver and kidney, with minor sites being the digestive organs and lung based on the relatively high amounts of radioactivity present as labeled endogenous compounds.

The actual distribution of radioactivity as ^{14}C amongst L-ET-18-OCH_3, peak 1 and peak 2 is tissue dependent. For example, in the liver more than 60% of the radioactivity was accounted for in peak 1 at 24–48 h post-dose compared to 20% in peak 1 for the kidney at this time.

Some tissues were analyzed for total radioactivity only. Approximately 10% of the total dose was accounted for in the skin. The concentration of EL (as μg equivalents) in the adrenal glands was about 11–12 μg eq/g while that in the sciatic nerve was about 5–7 μg eq/g. Negligible amounts of radioactivity were found in the bone marrow.

Discussion

L-ET-18-OCH_3 belongs to a class of alkyllysophospholipids (ALP) having antitumor activity [8–10], antiinflammatory [11] and antiparasitic [12,13] effects. Although racemic ET-18-OCH_3 was first synthesized over 30 years ago and its clinical antineoplastic activity was investigated in a small number of human studies [14], its use has been limited by hemolytic and gastrointestinal toxicities [15]. 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 [16].

Previously, we have shown that L-ET-18-OCH_3 is more cytotoxic *in vitro* than any of its most likely metabolites [5]. Thus the antitumor effect *in vivo* of TLC ELL-12 is probably due to L-ET-18-OCH_3. Once taken up into the plasma membrane of cells, ALPs can lead to inhibition of normal phospholipid metabolism. It has been suggested that one of the mechanisms for the selected toxicity to tumor cells is the slower metabolism of L-ET-18-OCH_3 in tumor tissues compared with normal tissues. Other mechanisms postulated for tumor cell growth inhibition and cytotoxicity include immunological modulation [17] and direct effects on signal transduction [18].

The purpose of this work was to study the activity, distribution and pharmacokinetics of TLC ELL-12 in MNU tumor-bearing rats. A significant delay in tumor growth was observed in the TLC ELL-12-treated groups compared to the untreated rats, although the effect of TLC ELL-12 at 25 mg/kg was not greater than the effect at the 12.5 mg/kg dose. Similar activity in MNU induced tumor has been observed by Berger *et al.* [6] at a dose level of 10 mg/kg of ET-18-OCH3. The MNU/Buff tumor cell line was less sensitive to L-ET-18-OCH_3 and TLC

ELL-12 (GI_{50}s of 32 and 63 µmol/l, respectively) than other tumor cell lines that we have tested *in vitro* [3]. Nevertheless, we were able to demonstrate the activity of TLC ELL-12 against these solid tumors in rats at doses we have found effective and well tolerated in other murine tumor models.

In our study, the half-life of L-ET-18-OCH_3 in tumor was estimated at 197 h (about 8 days), whereas the half-life of L-ET-18-OCH_3 in whole blood was 13.1 h. Tumor L-ET-18-OCH_3 levels increased through the first 16–24 h post-dosing and remained elevated at the terminal 48-h time point. The C_{max} of 23.2 µg/ml (44.3 µM) in the tumor was lower than the GI_{50} for TLC ELL-12 in this cell line, but is higher than the GI_{50} for L-ET-18-OCH_3. It is difficult analytically to separate 'free' and liposomal drug in tissues. Our values represent total (both free and liposome bound) drug in the tumor. However, as metabolites were detected in many tissues and we were able to demonstrate antitumor activity in this model, L-ET-18-OCH_3 is likely released from the liposome in the tumor.

The pharmacokinetic parameters in this experiment (three or four rats per time point) were very similar to those found in normal, cannulated Sprague-Dawley rats receiving the same dose of 12.5 mg/kg of TLC ELL-12 [5]. In both studies, there was very little metabolism in the blood and, in this study, there was very little metabolism in the tumor. Similar findings for free ET-18-OCH_3 have been reported by Magistrelli *et al.* [19].

In conclusion, TLC ELL-12 represents a novel agent that may be useful for the treatment of human neoplastic disease at non-toxic doses.

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