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TLC ELL-12

Oncolytic

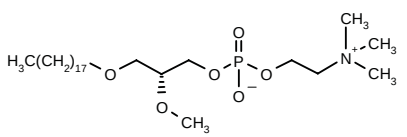
Liposomal ET-18-OCH_3
ELL-12

A liposomal formulation of the ether lipid ET-18-OCH_3

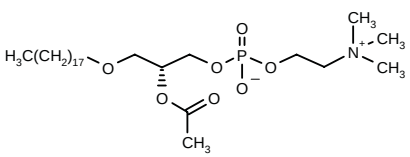
EN: 241095

Introduction

The synthetic ether lipid (EL) 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH_3, L-isomer) [I][1] is structurally similar to the naturally occurring platelet-activating factor (PAF) [II]. EL is also structurally similar to lysophosphatidylcholine (lyso-PC), but is much more resistant to metabolic turnover since it has an ether linkage in place of the readily hydrolyzable carboxylic ester linkage at the *sn*-1 position and a methoxy group in place of the hydroxy group (which is readily subjected to remodeling) at the *sn*-2 position. Indeed, the discovery that lyso-PC played a role in host defense mechanisms (1) was instrumental in the development of EL as an anticancer agent.



[I]



[II]

EL, unlike many other anticancer drugs, does not interact directly with cellular DNA and therefore is nonmutagenic. Elucidation of the mechanisms underlying the antiproliferative properties of ET-18-OCH_3 and the other alkyllysophosphocholines (ALPs) in general has been extensively studied. The abilities of these antitumor lipids to inhibit cell proliferation are derived from their interactions with cell membranes, where they spontaneously accumulate when added to cells. Their antiproliferative effects are cell selective (2), the basis of which is as yet unresolved. The multiplicity of sites of action and biochemical events that are affected when EL accumulates spontaneously in the plasma membranes of sensitive cells (2-4) have made it difficult to discern whether there is a single or primary mechanism of action that accounts for its growth-inhibitory effects. Many enzymes having lipid-binding sites are inhibited by EL. Prominent examples are CTP:phosphocholine cytidylyltransferase, the rate-limiting enzyme in phosphatidylcholine (PC) biosynthesis (5), and enzymes involved in the remodeling of the fatty acid composition of phospholipids (3). EL inhibits phosphatidylinositol 3-kinase, thus blocking the formation of several signaling phospholipids (6). However, there appears to be no correlation between the growth-inhibitory effect of EL and inhibition of the activity of protein kinase C (PKC) isoforms in several cell lines (3, 7, 8). By interfering with a number of cellular signaling pathways (3, 9), EL blocks the phosphorylation of extracellular signal-regulated kinases that are essential for cell proliferation and metabolism. Enhancement of cancer cell apoptosis by various mechanisms (10), activation of tumoricidal macrophages (11-13), inhibition of tumor cell invasion, reduction of metastases, inhibition of angiogenesis and promotion of tumor cell differentiation to macrophages or granulocytes are also involved in the antiproliferative activity of EL (2-4).

[1]The L-Isomer of ET-18-OCH_3 (which is the same as the *R* isomer) was used in the studies reported herein by The Liposome Company (TLC, now part of Elan Biopharmaceuticals). In many of the published biochemical studies referred to in this review article, however, racemic ET-18-OCH_3 (i.e., edelfosine) was used.

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A recent comparative study of ET-18-OCH_3 and a liposomal incorporated ET-18-OCH_3 (TLC ELL-12) showed that ET-18-OCH_3 triggers apoptosis by induction of caspase activation through the release of cytochrome c in a Bcl-X_L-sensitive manner but independently of the CD95 (APO-1/Fas) ligand/receptor system (14). This finding suggested that a formulation of ET-18-OCH_3 in liposomes could be a promising adjunct for the treatment of tumors in combination with myelosuppressive chemotherapeutic drugs and/or those that use the CD95-ligand/receptor system to trigger apoptosis.

Liposomal Formulations of ET-18-OCH_3

The clinical utility of ET-18-OCH_3 is significantly hampered by its acute hemolytic activity. This nonspecific toxicity is not surprising since the molecular structure of this amphipathic ether lipid closely resembles that of lyso-PC. The critical micellar concentration (cmc) of ET-18-OCH_3 in water is very low (~0.5 μM) (15). When the concentration exceeds the cmc, ET-18-OCH_3 is spontaneously incorporated into membranes. Under these conditions it is expected to disrupt endogenous lipid-lipid and lipid-protein packing; lyso-PC is known to disrupt PC vesicles. Unfortunately, to realize the therapeutic advantages of this compound dosages in excess of the cmc are required. To obviate the nonspecific lytic toxicity, a delivery system is needed that can lower the free drug's immediate availability, yet still allow the drug to ultimately gain access to the sites and tissues where its action can be therapeutic.

One approach to reduce the availability and hence toxicity of amphipathic compounds is to incorporate them into lipid-based carrier systems (*i.e.*, liposomes and lipid complexes). The molecular shape (*i.e.*, volume and cross-sectional area of the polar, including hydration, to the nonpolar regions) of lipids appears to play an important role in determining whether they form micelles, bilayers or inverted micelles (hexagonal II structure) (Fig. 1) (16, 17). The lamellar structure of liposomes results from the packing of lipids that have a cylindrical shape along their long axis. To obtain a lamellar structure in a mixture that contains a wedge-shaped, micelle-forming lipid, one must "compensate" for the micelle-forming lipid's inverted conical shape by adding a complementary shaped molecule (Fig. 1). In support of this concept, several investigators showed that mixtures of lyso-PC and cholesterol form a complex in which the lipids are packed in a lamellar structure (18-21). Therefore, it was proposed that mixing ET-18-OCH_3, which forms a micellar structure in the absence of another lipid, with lipid(s) of a complementary molecular shape may yield a stable lamellar structure, from which the rate of transfer of ET-18-OCH_3 molecules to erythrocyte membranes would be reduced. Indeed, it was found that the lytic nature of ET-18-OCH_3 was markedly reduced by complexing it with cholesterol and incorporating it into bilayers prepared with phospholipids (see below).

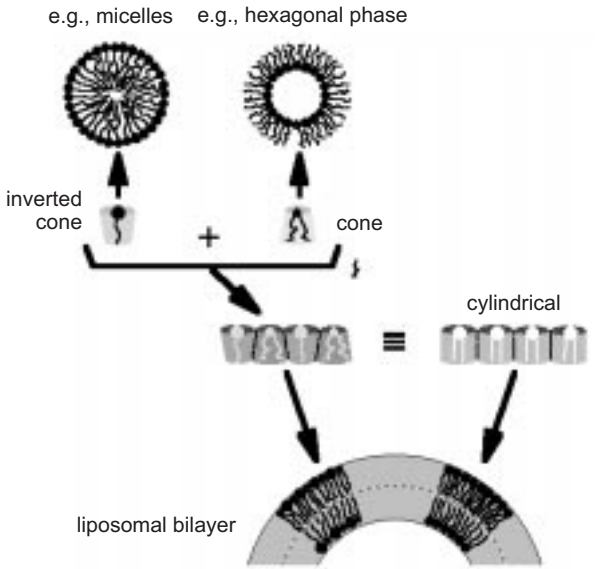

Fig. 1. The phase adopted when lipids self-assemble is dictated by the packing parameter (the relationship between the hydrocarbon volume and the polar head group surface area) of the individual lipids. The combination of a lipid that normally forms a micelle, such as ET-18-OCH_3, with a lipid of complementary molecular shape can result in a lamellar (bilayer) structure.

Packing parameters are, of course, dependent on many factors in addition to the molecular shape, such as hydration, electrostatics, attractive van der Waals forces, steric hindrance, *etc.* In order to assess complementarity of molecular shape in an experimental manner, Langmuir monolayer films were used (22). The mean molecular area per molecule (MMAM) for monolayers comprised of binary mixtures of ET-18-OCH_3 and another lipid (cholesterol, dioleoylphosphatidylethanolamine [DOPE], 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine [POPC] or 1,2-dioleoyl-*sn*-glycero-3-phosphocholine [DOPC]) was measured. Shape complementarity was considered to occur when the MMAM for the lipid mixture was found to be significantly less than the value predicted based on simple additivity of the MMAM of each pure compound. Cholesterol and DOPE were chosen in particular because both molecular species have similar theoretical packing parameters and both have been shown to form lamellar structures when combined with lyso-PC (18, 19).

For binary mixtures, the greatest degree of molecular shape complementarity was observed with cholesterol; the order of complementarity with ET-18-OCH_3 was cholesterol > DOPE > POPC ≈ DOPC (Fig. 2). The higher degree of shape complementarity with cholesterol compared with DOPE was surprising. The reduction in MMAM was maximal for all mixtures at approximately 40 mol% ET-18-OCH_3.

To examine whether a lamellar structure is formed when the various lipids were mixed with 40 mol% ET-18-OCH_3, the line shape (chemical shift anisotropy) of the ^{31}P-NMR signal, which is known to be an indicator of

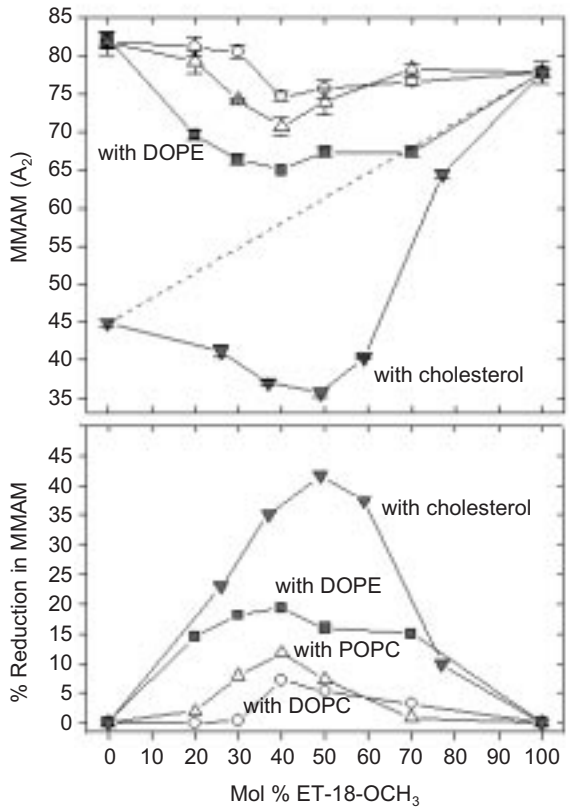

Fig. 2. Upper panel: Mean molecular area per molecule (MMAM) as a function of ET-18-OCH_3 content. The monolayers were formed from ET-18-OCH_3 mixed with the indicated lipids. Data are the average of 3 experiments ± SD. The dashed line represents the expected values (A_0) for cholesterol/ET-18-OCH_3 mixtures based on simple additivity of the individual values of MMAM; $A_0 = X_1 \cdot A_1 + X_2 \cdot A_2$ where X is the mole fraction of each component and A_n is the MMAM measured for the individual lipids 1 and 2. The magnitude of the deviation from this curve indicates the degree of shape complementarity. Lower panel: Percent reduction in MMAM as a function of ET-18-OCH_3 equals 100 x $[(A_0 - A_{exp})/A_0]$. (Reproduced with permission from reference 22.)

phospholipid polymorphism (23), was analyzed in aqueous dispersions prepared from the mixed lipid films. To assess hemolysis, large unilamellar vesicles (LUVs) of approximately 100-nm diameter were prepared by extrusion through polycarbonate filters; these structures are expected to have only a single bilayer. The hemolytic activity (H_{50} value) of the LUVs followed the same trend as the reduction in MMAM observed in the monolayer films; the MMAM and H_{50} value were the lowest for ET-18-OCH_3/cholesterol mixtures, followed by ET-18-OCH_3/DOPE mixtures and then by mixtures with POPC or with DOPC, which were comparable. For ET-18-OCH_3/cholesterol mixtures made at higher drug mole percentages (*i.e.*, 50 and 60 mol% ET-18-OCH_3), the ^{31}P NMR signal revealed the presence of two populations – a lamellar and a micellar population. Dispersions made from ET-18-OCH_3 and cholesterol at these higher molar ratios displayed the same hemolytic activity as the free drug.

The conclusion of this study is that a lipid, such as cholesterol, with a molecular shape complementary to that of ET-18-OCH_3 should be included in the design of liposomes used to deliver ET-18-OCH_3 to tumor cells.

To increase tumor accumulation of the drug, lipids that are known to enhance the circulation lifetime of liposomes must be included. Table I shows a partial listing of the lipid formulations that were tested (24). For the series of formulations listed, a negatively charged lipid species (various phosphatidylethanolamines containing glutaric acid (GA) coupled via an amide linkage to the head group) and a zwitterionic PC species are included. The negatively charged lipid was included to help avoid aggregation and increase circulation lifetime (25). Variation in the extent of acyl chain saturation of PC and PE-GA greatly affected the hemolytic activity of the resulting liposomes. The hemolytic activity decreased significantly when both the zwitterionic and negatively charged lipids bore dioleoyl chains. Interestingly, the formulation containing DOPE was much less hemolytic than that containing DOPC as the neutral species, as shown in the last two entries of Table I. With the binary systems mentioned earlier, ET-18-OCH_3 in combination with DOPE was much less hemolytic than when it was combined with DOPC.

When the reductions in hemolytic activity and membrane permeability for various formulations are compared (24), a correlation is observed that supports the hypothesis that with more ideal packing between lipids, the extent

Table I: Hemolytic activity of ET-18-OCH_3 (EL) formulations.

Formulation	Mole ratio	HI_5 (μM)	(n)
Free EL	--	5.6 ± 0.9	3
EPC:cholesterol:DPPE-GA:EL	**1**:3:1:**5**	9	1
	3:3:1:**3**	14.1 ± 3	2
	4:3:1:**2**	19	1
	5:3:1:**1**	33	1
EPC:cholesterol:POPE-GA:EL	**1**:3:1:**5**	14	1
	3:3:1:**3**	20	1
	4:3:1:**2**	31.5 ± 2.3	2
	5:3:1:**1**	31	1
EPC:cholesterol:**DPPE-GA**:EL	4:3:1:2	19	1
EPC:cholesterol:**POPE-GA**:EL	4:3:1:2	31.5 ± 2.3	2
EPC:cholesterol:**DOPE-GA**:EL	4:3:1:2	140	1
DSPC:cholesterol:DOPE-GA:EL	4:3:1:2	22	1
POPC:cholesterol:DOPE-GA:EL	4:3:1:2	45	1
EPC:cholesterol:DOPE-GA:EL	4:3:1:2	140	1
DOPC:cholesterol:DOPE-GA:EL	4:3:1:2	250 ± 25	5
DOPE:cholesterol:DOPE-GA:EL	4:3:1:2	640 ± 60	2

Listing of some formulations is repeated in order to show trends. HI_5: concentration at which 5% hemolysis is produced; EL: ET-18-OCH_3; EPC: egg phosphatidylcholine; PE-GA: phosphatidylethanolamine (PE) with glutaric acid derivatization of the head group; DSPC: distearoylphosphatidylcholine; POPC: 1-palmitoyl-2-oleoyl-PC; DOPC: dioleoyl-PC; DOPE: dioleoyl-PE.

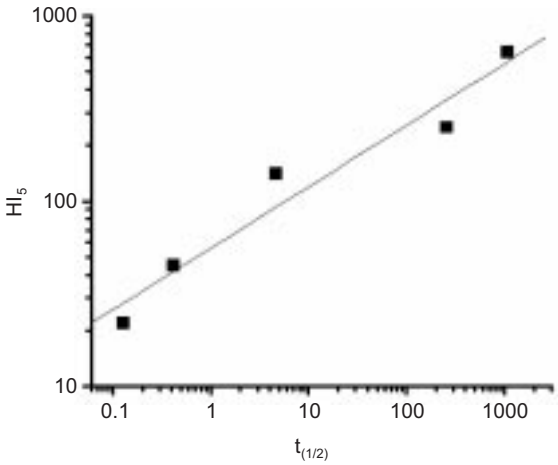

Fig. 3. Carboxyfluorescein leak as expressed at $t_{1/2}$ *vs.* hemolytic activity, which is expressed as the EL concentration at which 5% hemolysis was noted (HI_5). The line represents a linear fit of the data. (Data were taken from reference 24.)

of ET-18-OCH_3 transfer from liposomes, and therefore the extent of hemolysis, is reduced (Fig. 3). The least two hemolytic systems *in vitro* (the last two entries in Table I) were tested *in vivo*, and both were acceptable in terms of hemolysis. However, the DOPC/cholesterol/DOPE-GA/EL formulation appeared to be more effective in an *in vivo* murine model of melanoma (B16/F10) (data not published). This formulation, which was designated as TLC ELL-12, was taken forward for pharmaceutical development.

Pharmacological Actions

The *in vitro* growth inhibitory activities of ET-18-OCH_3 and TLC ELL-12 have been examined using the sulforhodamine B (SRB) assay in several tumor cell lines (26-28). The concentrations required to inhibit the growth of the cells by 50% compared with controls (GI_{50}) after 72 h of incubation are shown in Table II. The GI_{50} values were 1.2-2.2-fold greater for TLC ELL-12 than for free ET-18-OCH_3. The normal cell line investigated (NIH 3T3 fibroblasts) was much less sensitive than the series of tumor cells.

Although the incorporation of ET-18-OCH_3 into liposomes slightly attenuates its growth inhibitory effects *in vitro*, this is not the case *in vivo*. TLC ELL-12 has an enhanced or equivalent antitumor activity compared with free ET-18-OCH_3. This has been demonstrated in several *in vivo* tumor models, including mouse Lewis lung tumor, B16 melanoma, P388 leukemia and a human DU 145 prostate tumor (29, 30). Figure 4 shows the efficacy of TLC ELL-12 in a B16 murine melanoma model. The number of lung metastases is reduced significantly after treatment with TLC ELL-12 at a dose of 6.25 mg/kg given every other day for 5 doses. As with other agents that act via signal transduction or immunomodulatory

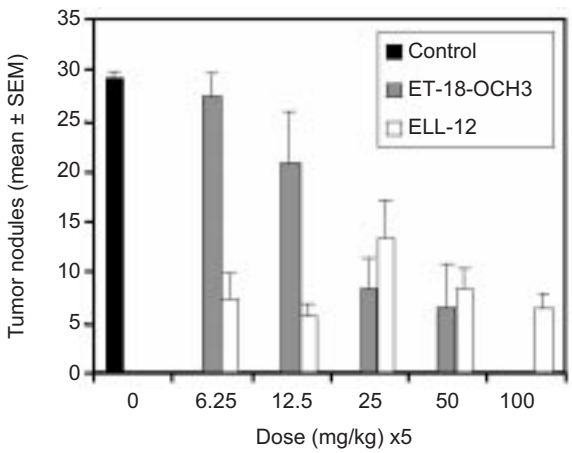

Fig. 4. Inhibitory effects of ET-18-OCH_3 or TLC ELL-12 against B16/F10 lung tumors in mice. Groups of mice (10/group) were injected i.v. with 5 x 10^4 B16/F10 cells. Treatment was administered i.v. on days 10, 12, 14, 16 and 18 at dose levels of 6.25-100 mg/kg. Mice were sacrificed on day 22, lungs were removed and fixed in formalin, and tumor nodules were counted "blind" using an inverted microscope. When there were more than 30 nodules per lung, the value was entered as 30 for the calculation of means. (Redrawn from reference 29.)

Table II: Growth inhibition (50%) concentration (GI_{50}) of free ET-18-OCH_3 and TLC ELL-12.

Cell line		GI_{50}, μM ET-18-OCH_3	TLC ELL-12
A549	Human small cell lung cancer	6.5	13.7
MCF 7	Human breast carcinoma	16.8	28.8
L1210	Murine leukemia	3.9	4.4
P388	Murine leukemia	5.0	7.2
U937	Human leukemia	1.4	1.6
HL-60	Human leukemia	1.9	2.9
HT 29	Human color tumor	6.0	7.0
Ovcar 3	Human ovarian carcinoma	6.2	14.1
DU 145	Human prostate carcinoma	14.0	17.3
NIH 3T3	Normal fibroblasts	111.0	149.0

Table III: Highest nonlethal and minimum lethal doses of TLC ELL-12 in various species.

Species	Regimen	Highest nonlethal dose (mg/kg/day) M	Highest nonlethal dose (mg/kg/day) F	Minimum lethal dose (mg/kg/day) M	Minimum lethal dose (mg/kg/day) F
Mouse	Single, slow, i.v. bolus dose	100	100	150	150
Mouse	Slow i.v. bolus dose once daily for 5 days	75	75	100	100
Rat	Single 20-min i.v. infusion	37.5	112.5	112.5	150
Rat	I.v. infusion at 25 mg/kg/h once daily for 5 days	75	37.5	>75	75
Rat	I.v. infusion at 25 mg/kg/h once daily for 14 days	6.25	12.5	12.5	37.5
Dog	Single i.v. infusion at 25 mg/kg/h	< 6.25	< 6.25	< 6.25	< 6.25
Monkey	Rising dose i.v. infusion at 1-4 mg/kg/h once daily for 5 days	ND	>37.5	ND	>37.5
Monkey	I.v. infusion at 1-4 mg/kg/h once daily for 9-14 days	>25	>25	>25	>25

ND = not done

mechanisms, there is no clear benefit to increasing the dose above a therapeutic threshold, in this case, approximately 6-12 mg/kg.

Toxicology

The toxicology of TLC ELL-12 has been studied in mice, rats, dogs and cynomolgus monkeys receiving daily intravenous infusions for up to 14 consecutive days (31, 32). The minimum lethal doses (considered likely or possibly related to treatment) for all toxicology studies are shown in Table III. The minimum lethal single intravenous dose of TLC ELL-12 in mice and rats was greater than 100 mg/kg. In repeated dose studies in rats, deaths were considered to result from vascular inflammation and thrombosis at the intravenous catheter site (common in animals surgically catheterized for intravenous infusion studies), which was exacerbated by TLC ELL-12. Dogs did not tolerate TLC ELL-12, probably because of effects of the drug on their platelets (see below). Male monkeys in the high dose (25 mg/kg/day) group of the 14-day monkey study showed a slight decrease in body weight.

Intravascular hemolysis was considered to be one of the major dose-limiting toxicities in the early studies with ET-18-OCH_3 in humans. As stated above, TLC ELL-12 was developed in an effort to reduce or eliminate this toxicity. *In vitro* blood compatibility studies with whole blood from rats, dogs and human volunteers showed that hemolysis in the presence of TLC ELL-12 was much reduced compared with free ET-18-OCH3, being negligible at a concentration of 400 μg/ml and slight at a concentration of 1600 μg/ml.

Effects on platelets, including aggregation, vascular inflammation, thrombosis and thrombocytopenia were seen in rats, dogs and monkeys. The dog did not tolerate intravenous infusion of TLC ELL-12. All dogs that received TLC ELL-12 at doses of 6.25 mg/kg or higher showed clinical signs within minutes after initiation of treatment. These signs included injected sclera, dilated pupils, retching, bloody vomiting and diarrhea, rapid, shallow and labored respiration, hypoactivity, recumbency and cold-to-touch. All dogs died or were sacrificed within several hours following a single dose.

In vitro platelet aggregation studies utilizing impedance aggregometry with TLC ELL-12 or ET-18-OCH_3 suggested that the dog was peculiarly sensitive to ET-18-OCH_3. There were no effects of TLC ELL-12 (at concentrations up to 500 μg/ml) on aggregation of platelets in whole blood from rats and monkeys, but there was a positive response in all dogs tested at TLC ELL-12 concentrations at and above 10 μg/ml (well below the concentrations detected in the blood of dogs during the infusion of TLC ELL-12). This led to the conclusion that in dogs the initiation of platelet aggregation in the systemic circulation was the event that resulted in lethal toxicity.

TLC ELL-12 did not appear to be myelosuppressive. There were no histologic findings in the bone marrow of rats and there was myeloid hyperplasia in the bone marrow of monkeys given 25 mg/kg/day for 8 days. White blood cell counts of rats and monkeys treated with high doses of TLC ELL-12 were generally comparable to controls or increased.

TLC ELL-12 did not have an effect on neurologic function based on a functional observational battery performed on rats after receiving 14 daily doses of test material. TLC ELL-12 was negative in the bacterial reverse mutation assay (Ames test) and the mammalian erythrocyte micronucleus test (27).

Pharmacokinetics

A study of the distribution of ET-18-OCH_3 after administration of TLC ELL-12 was conducted in Buffalo rats with transplantable *N*-methyl-*N*-nitrosourea (MNU)-induced tumors (31). Initial work showed that a concentration of 20-25 μg/ml could inhibit the growth of these cells *in vitro* and that a dose of 12.5 mg/kg of TLC ELL-12 could inhibit the growth of the tumor *in vivo*. To determine the blood and tumor levels of ET-18-OCH_3 after a therapeutic dose of TLC ELL-12, female Buffalo rats bearing MNU tumors were given 12.5 mg of ET-18-OCH_3/kg as TLC ELL-12 containing radiolabeled 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-[*N*-methyl-^{14}C]-phosphocholine by i.v. push injection (Fig. 5). The total radioactivity was determined by liquid-scintillation

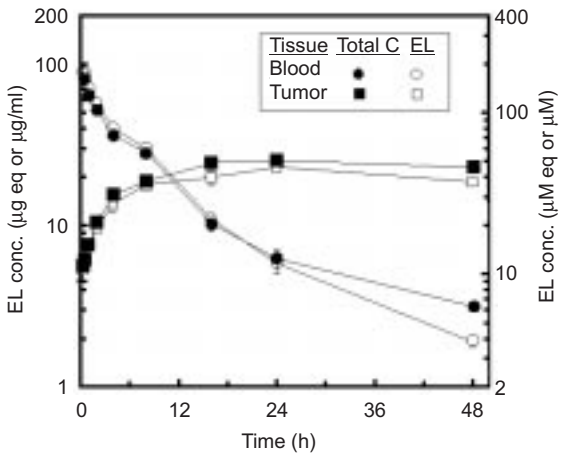

Fig. 5. Whole blood and tumor concentrations of EL (by specific HPLC assay) and total radioactivity in rats bearing MNU mammary carcinoma and treated with a single 12.5 mg/kg i.v. bolus of TLC ELL-12.

counting. ET-18-OCH_3 concentrations were determined using an HPLC method with radiochemical detection. This chromatographic method was capable of resolving the parent drug from potential radioactive metabolites and from classes of endogenous compounds that were likely to become labeled as a consequence of such metabolism. The latter included polar compounds (choline, phosphocholine) and phospholipid intermediary metabolites. The concentration-time courses were evaluated in whole blood, tumor and other tissues (n = 3-4 rats per point). The peak blood concentration of EL was approximately 90 µg/ml and the area under the plasma concentration-time curve (AUC_{0-48h}) was 716 µg·h/ml (33). Visual inspection of the blood concentration *vs.* time curve suggests that ET-18-OCH_3 pharmacokinetics are multiexponential, with distributional events occurring over the first 4-8 h and with a terminal half-life in whole blood of ~13 h. The maximum concentration (~23 µg/g) of parent drug was attained in the tumor tissue during the first 24 h post-dose and persisted up to the last time point (48 h) with little evidence of metabolism. The AUC_{0-48h} in tumor tissue was 928 µg·h/ml. As with other liposomal drugs, the tissue EL concentrations were highest in the spleen and liver, and in the lungs, kidneys and organs of the gastrointestinal tract. The percent of radioactivity present in labeled endogenous compounds (metabolites) was notable in the liver and kidney.

In summary, these studies suggest that the ET-18-OCH_3 delivered in TLC ELL-12 undergoes very little excretion and slow metabolism, and that its pharmacokinetics are dominated by distributional clearance (*i.e.*, uptake into tissues). The persistence of ET-18-OCH_3 in tissues, particularly in tumor tissue, suggests that relevant levels, once attained, could theoretically be maintained with relatively infrequent maintenance dosing.

Clinical Studies

A phase I trial of TLC ELL-12 in patients with refractory solid tumors is currently in progress (33). Thus far, 23 patients have received 5 consecutive daily doses ranging from 0.5-16 mg/kg infused intravenously at 1-4 mg/kg/h and repeated every 3 weeks. TLC ELL-12 has been well tolerated. No dose-limiting toxicity has been seen and no patient has experienced myelosuppression. An infusion rate-related reaction (similar to that seen with other liposomal agents) at 4 mg/kg/h responded to slowing of the infusion or premedication. One patient with heavily pretreated, rapidly progressive metastatic melanoma had disease stabilization during 7 cycles (~6 months) of TLC ELL-12 treatment at a dose of 7 mg/kg. Another patient with metastatic renal cell carcinoma treated at a dose level of 12 mg/kg has had a complete response, now sustained for 6 months. Detectable levels of ET-18-OCH_3 circulate for at least 7 days at doses above 5 mg/kg. After single doses of 9-12 mg/kg, the AUC_{0-48h} ranged from 528-924 µg·h/ml, which is comparable to that seen with a therapeutic dose in the rat. The pharmacokinetic results suggest that doses of 9-12 mg/kg are in the therapeutic range and that extended dosing intervals may be feasible without sacrificing potentially therapeutic levels of drug exposure.

Manufacturer

Elan Biopharmaceuticals (US).

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