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Research Paper

A Parenteral Econazole Formulation Using a Novel Micelle-to-Liposome Transfer Method: *In Vitro* Characterization and Tumor Growth Delay in a Breast Cancer Xenograft Model

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Purpose. The purpose of this study was to develop a parenteral liposomal formulation of econazole, a poorly water-soluble compound not previously available in an intravenous form. We are investigating econazole as an anticancer agent based on its unique mechanism of action to which cancer cells are preferentially sensitive. An intravenous formulation of econazole was desired for preclinical toxicity and efficacy studies of econazole.
Methods. Liposomal econazole was prepared using a novel micelle exchange technique to incorporate the drug into the lipid bilayer of pre-formed liposomes using a poly(ethylene) glycol-linked phospholipid, distearoyl phosphatidylethanolamine (DSPE-PEG). This method allowed for stable and efficient drug incorporation into DPPC and DMPC liposomes at a final drug:lipid ratio of 0.05 (*w*/*w*) and increased solubility in saline from <0.1 to 5 mg/ml.
Results. Stability over 14 days at 4°C in buffer was demonstrated as well as *in vitro* plasma stability at 37°C. Plasma elimination studies of micelle-loaded liposomal econazole showed a half-life of approximately 35 min and plasma AUC of 281 μg/ml min. In MCF-7 human breast cancer xenografts in Rag2M mice. Liposomal econazole did not induce significant hepatoxicity, renal toxity or weight loss compared to empty liposomes. Tumor growth was slightly delayed in liposomal econazole-treated mice, with ~10-day lag time to reach 300 mm^3 compared to vehicle controls.
Conclusions. The micelle transfer method provided an efficient means of preparing liposomal econazole suitable for intravenous administration. Liposomal econazole was successfully administered to tumor bearing mice at 50 mg/kg, and no significant toxicities attributable to econazole were observed.

KEY WORDS: cancer; econazole; liposomes; micelles; tumor studies.

INTRODUCTION

Econazole is an imidazole topical antifungal agent (Fig. 1) presently under investigation for its anticancer effects. Its mechanism of action involves stimulating calcium release from stores in the endoplasmic reticulum while concurrently blocking calcium influx into the cell. In this manner, econazole depletes cellular calcium stores and leads to inhibition of protein synthesis and cell death (1,2). Activated cells, such as proliferating cancer cells, are preferentially sensitive to this mechanism. Econazole has shown cytotoxicity in several human cancer cell lines including breast cancer (3,4), osteosarcoma (5), PC3 prostate cancer cells (6), colon cancer (7) and myeloid leukemia cells (8,9). Preliminary *in vivo* studies showed that direct injection of econazole into MCF-7 human breast cancer xenografts grown in mice caused a delay in tumor growth compared to DMSO vehicle controls (10). However, at the present time, there is no commercially available parenteral dosage form for econazole. Furthermore, econazole is poorly water soluble and highly protein bound (11), which limits achieving an efficacious dose in tumors. Therefore, if econazole is to be developed as an anticancer drug, a formulation suitable for systemic use would be desirable. The purpose of the present study was to develop a well-tolerated liposomal formulation of econazole for preliminary preclinical studies.

Given the poor water solubility of econazole (<0.2 mg/ml at ≥ 37°C and <50 μg/ml at 23°C in pH 7.0 buffer), this drug was not suitable for passive encapsulation of econazole into the aqueous liposomal interior. Although econazole has protonizable moieties that make it a candidate for pH gradient loading (12), we found the trapping efficiency of econazole to be very low (E. Wasan, unpublished observations). To address these issues, we devised a novel micelle-based liposome loading method. This method uses long chain

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hydrophilic polymer-linked lipids, such as distearoyl phosphatidylethanolamine-linked poly(ethylene) glycol (DSPE-PEG_{2000}) that can form micelles in solution above a critical micelle concentration (CMC). For example, the CMC of DSPE-PEG_{2000} at ambient temperature in isotonic solution is reported to be approximately 0.5 μM (13). Additionally, PEG lipids have several functional roles in liposomal formulations, e.g., to increase liposome circulation time (14,15) and to prevent liposomal aggregation (16,17). Econazole was solubilized in DSPE-PEG_{2000}, which formed micelles, followed by passive partitioning into pre-formed liposomes, generating a 50-fold improvement in solubility of econazole compared to physiological saline [HEPES-buffered 150 mM NaCl (HBS), pH 7]. The anticancer properties of the resulting formulation were assessed *in vivo* using a human MCF-7 breast cancer xenograft model. To the best of our knowledge, this represents the first formulation of econazole suitable for intravenous use and the first application of systemically administered econazole for the treatment of cancer (10,16).

MATERIALS AND METHODS

Materials

Econazole nitrate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dipalmitoyl phosphatidylcholine (DPPC), dimyristoyl phosphatidylcholine (DMPC) and distearoyl phosphatidylethanolamine-poly(ethylene glycol)$_{2000}$ (DSPE-PEG) with an average PEG molecular weight of 2000 were purchased from Avanti Polar Lipids (Albaster, AL, USA). Tritiated cholesteryl hexadecyl ether ([^{3}H]-CHE) and [^{14}C]-distearoyl phosphatidylethanolamine-poly(ethylene glycol) ([^{14}C]-DSPE-PEG_{2000}) were purchased from Perkin Elmer (Boston, MA, USA). Whatman Nuclepore 200-, 100- or 80-nm filters were used in a 3 ml Lipex Extruder, all from Northern Lipids (Vancouver, BC, Canada). Sephadex G-50 and Sepharose CL-4B size-exclusion chromatography beads were also purchased from Sigma. Other reagents were either from Sigma or Fisher Chemicals (Fairlawn, NJ, USA). All solvents were HPLC grade. Water was prepared by a reverse osmosis system (MilliQ) and filtered (0.22 μm) prior to use. Buffers were also filtered prior to use (0.22 μm).

Econazole UV Spectrophotometric Assay

Econazole was dissolved in methanol up to a concentration of 25 mg/ml and a characteristic absorption peak was observed in the ultraviolet range (λ = 271 nm). Econazole experimental samples were quantified by comparison with a standard curve ($r^2 \geq 0.995$) with a linear range of 0.05–1.0 mg/ml. For liposomal econazole, the absorbance readings of empty liposomes were subtracted from liposomal econazole samples as background, and samples were typically diluted at 1:10 (*v*/*v*) in methanol (to clarity) prior to analysis to solubilize the liposomes and econazole.

Liposome Preparation

Lipid constituents were weighed out in the desired molar ratio and solubilized in chloroform. A nonexchangeable, non-metabolized radioactive lipid tracer, [^{3}H]-CHE (~0.5 μCi/μmol) was added to the dissolved lipids for lipid quantitation post extrusion (18). The lipid solution was dried to a thin film under N_2 gas, followed by hydration with HEPES-buffered saline (HBS: 25 mM HEPES, 150 mM NaCl, pH 7.2) at 50°C for DPPC and 37°C for DMPC for 1 h with frequent vortexing. Five cycles of freeze and thaw were then performed with liquid N_2 and a waterbath set at 37°C for thawing DMPC liposomes and 50°C for DPPC liposomes. The sample was then extruded at 50°C (DPPC) or 37°C (DMPC) by passing the sample ten times through two stacked polycarbonate filters of 200-nm pore size with a Lipex Thermobarrel Extruder (19). Quasi-elastic light scattering (Nicomp 270, Particle Sizing Systems, Santa Barbara, CA) was used to determine mean diameter and particle size distribution of the liposomes and micelles.

Liposomal Formulations

Econazole was incorporated into the lipid bilayer during liposome formation followed by exchange of DSPE-PEG_{2000} into the outer leaflet (Fig. 3A, thin film/extrusion method), or econazole was incorporated into the outer leaflet of the lipid bilayer by exchange of DSPE-PEG/econazole micelles into pre-formed liposomes (Fig. 3B, micelle loading method). In the first case, DMPC or DPPC and econazole were mixed during the thin film stage of liposome preparation followed by extrusion, as described above. Separately, DSPE-PEG_{2000} was solubilized in HBS/ethanol (2:1 *v*/*v*), heated to 37°C (DMPC) or 50°C (DPPC) until clear micelles were formed (~15-nm diameter) and then added to the warmed liposomes at 5 mol% and final ethanol concentration at 4.3% (*v*/*v*) (Fig. 3A). For the micelle loading method, DMPC or DPPC liposomes were prepared first by extrusion as described above. Micelles of DSPE-PEG_{2000} with or without econazole were prepared by first solubilizing the lipid and/or drug in 100% ethanol, followed by addition of HSB (pH 7.2) such that the ratio of HBS/ethanol was 2:1 (*v*/*v*). The cloudy suspension was then mixed and warmed (50°C) for 15–30 min until clear. By dynamic light scattering, the micelles formed were ~15 nm diameter. Liposomes and micelles were then combined by simple mixing at 37°C (DMPC) or 50°C (DPPC) for 60 min, followed by storage at 4°C overnight to complete equilibrium transfer. The final econazole concentration was 5 mg/ml, lipid concentration was 100 mg/ml and the lipid ratio (DMPC or DPPC: DSPE-PEG_{2000}) was 95:5 (mol:mol). The ethanol concentration was 4.3% (*v*/*v*) in the initial mixture of liposomes and DSPE-PEG/econazole micelles.

Analysis of Drug Loading

Liposomes were incubated with the micelles of DSPE-PEG ± econazole for 1 h at 37°C (DMPC-containing liposomes) or 50°C (DPPC-containing liposomes). To separate liposome-associated econazole from free econazole, 500 μl of sample were added (in triplicate) at each timepoint to a 10-ml size exclusion Sepharose CL4B column pre-equilibrated in HBS (pH 7.2), in which the liposomes elute in the void volume. The liposome-containing eluate was analyzed by UV spectroscopy for econazole as described above. Lipid concentration was measured by triplicate scintillation counting of the

[^{3}H]-CHE lipid tracer, and the drug:lipid ratio (*w*/*w*) was calculated. For each sample type, at least three independent liposome preparations were analyzed, and the mean drug:lipid ratio at each time point is reported. Free econazole could not be eluted from this column due to low drug solubility in buffer. Total recovery of liposomal econazole from the column was 95 ± 8% by mass balance.

Stability Analysis in Plasma

For stability studies in plasma, three separate preparations of micelle-loaded liposomal econazole were made with trace [^{14}C]-CHE and [^{3}H]-DSPE-PEG$_{2000}$ as described above using DMPC or DPPC as the main lipid constituent. The liposomes were mixed with human plasma at a ratio of 1:3 (*v*/*v*) and incubated at 37°C for 30 min. The plasma was applied to a 10 ml CL4B size exclusion chromatography column equilibrated in HBS and at least 25 fractions were collected at a rate of 0.7 ml/min to determine if econazole and PEG lipid were associated with liposomes or with plasma protein-containing fractions. Each fraction was analyzed in triplicate for [^{14}C]-CHE as a measure of the liposome-containing fractions, for econazole, for total phosphorus by the method of Fiske and Subbarow (20) as a measure of phospholipid concentration (e.g., DPPC and DSPE-PEG), and for total protein. The three measures were averaged for each parameter, and these means were combined from the 3 different batches of liposomes for the data represented in the figures. Protein analysis was performed by visible spectrophotometry (λ = 562 nm) using the bicinchoninic acid assay (Sigma) and compared to a triplicate standard curve of bovine serum albumin (linear range = 0–100 μg/ml, $r^2 \geq 0.995$). The presence of empty liposomes, DSPE-PEG/econazole micelles or drug-loaded liposomes did not affect the fractional distribution of plasma proteins on the column, rather, the liposomes (e.g., fractions 5–8) and micelles or protein fractions (e.g., fractions 10–14) were consistently distinct from each other. Likewise, the fractional distribution of the liposomes was not affected by the presence of econazole (in the liposomes or in DSPE-PEG micelles) or plasma proteins. Econazole analysis was by liquid–liquid extraction consisting of fraction sample, H_2O and ethyl acetate at a ratio of 1:1:6 (*v*/*v*/*v*). Samples were vortex mixed for 5 min and centrifuged at 10,000 × *g* for 5 min. The top organic layer was removed, dried under N_2 gas and reconstituted in 100 μl methanol. The econazole assay was performed as described above. Background consisted of the corresponding extracted fractions of empty liposomes.

Pharmacokinetics

For analysis of pharmacokinetic samples, 200 μl of plasma were extracted 2 times with 3 volumes of ethyl acetate and 2 volumes of 0.1 M NaOH, with vortexing for 15 min for each extraction, and centrifugation at 1,500 × *g* for 10 min to separate organic and aqueous phases. The combined organic phases were dried at 60°C under vacuum in a vortex evaporator for approximately 20 min. The dried extract was reconstituted in 100–200 μl acetonitrile and centrifuged to remove any residue. The supernatant (10 μl) was injected onto the HPLC by autoinjector the same day. The HPLC column was a NovaPak RP-18 (C18, 75 × 46 mm, 4 μm) and the mobile phase was acetonitrile: 10 mM ammonium formate + 20 mM diethylamine (64:36) run at a flow rate of 1 ml/min at 28°C column temperature. UV detection (λ = 271 nm) was performed with a photodiode array detector (Waters 996). Quantitation of samples was performed using an external standard curve of econazole prepared in triplicate in mouse plasma, using the same extraction method as the samples ($r^2 > 0.995$, linear range 20–250 μg/ml, limit of detection = 10 μg/ml). Extraction efficiency was ~90% across the concentration range. Pharmacokinetic noncompartmental analysis was performed using WinNonLin version 1.5 software (Scientific Consulting). AUCs were generated using the mean concentrations (n = 6) at each timepoint because each data point was generated from an individual animal rather than by serial sampling, due to the small plasma volume obtainable from mice. Comparison between treatment groups was performed at each timepoint using MicroCal Origin software with a two-tailed, paired Student's t test where significance was set at $p \leq 0.05$.

Multidose Tolerability Studies in Mice

Single dose and multi-dose tolerability studies were performed on Rag2M female mice at 50 mg/kg econazole dose via intravenous injection into the lateral tail vein at a volume of 200 μl/20 g mouse once (single dose) or every other day for six doses (multidose). The care, housing and use of animals were performed in accordance with the Canadian Council on Animal Care Guidelines. Four formulations were tested in the single-dose study, comparing DPPC and DMPC liposomes containing econazole prepared by the thin film/extrusion method *vs* the micelle-loaded form. In all cases the final lipid ratio was 95:5 (mol/mol) (DPPC or DMPC: DSPE-PEG$_{2000}$) and the drug:lipid ratio was 0.05 (*w*/*w*). The vehicle controls consisted of the corresponding liposomes not containing econazole. For the multidose study, only the DPPC-based liposomal econazole formulations were assessed prior to efficacy studies, because their stability was greater than the DMPC-based liposomes.

For both the single and multi-dose studies, mice (n = 3/group) were weighed daily during the drug administration period and for 14 days after the last dose. Observation of appearance and behavior also continued for 14 days after the last dose and was scored by a certified animal technician to ascertain morbidity using institutional protocols. At the end of the study, the mice were terminated by CO_2 inhalation and blood was collected immediately by cardiac puncture. The blood was allowed to clot for 1 h, and then the serum was separated by centrifugation 1,000 × *g* for 15 min. Serum was frozen in liquid N_2 and stored at −20°C until shipment to Central Laboratory for Veterinarians (Surrey, BC, Canada) for analysis of liver enzymes (alkaline phosphatase, AST, ALT, GGT, bilirubin, sorbital dehydrogenase), electrolytes, BUN and creatinine.

Efficacy Study in MCF-7 Xenografts in Mice

Mice received estradiol as 60-day slow-release subcutaneous pellets 1 day prior to tumor cell inoculation. The mice were injected with 1 × 10^7 MCF-7 cells (American Type

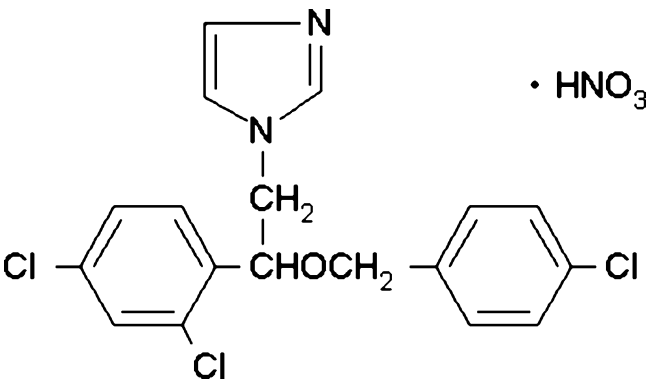

Fig. 1. Chemical structure of econazole: [(dichloro-2,4 phenyl)-2(chloro-4 benzoyloxy)-2 ethyl]-1 imidazole nitrate.

Culture Collection, ATCC) subcutaneously. In the preliminary direct injection studies, econazole was freshly prepared in 100% DMSO. The mice were injected intratumorally daily for four doses of 65 or 100 mg/kg or vehicle control, beginning on day 41 post tumor inoculation when tumors were approximately 50–80 mm^3. Tumors were measured daily until day 64. In the liposomal formulation efficacy studies, the mice were randomized to treatment groups then injected with 50 mg/kg of liposomal econazole or empty liposomes via the lateral tail vein (as 0.2 ml per 20 g mouse weight) once the tumors reached approximately 50 mm^3, with dosing every other day excluding weekends for a total of six doses, starting at day 17 post-tumor inoculation. Tumors were measured daily until day 60, at which time the mice were sacrificed and serum was collected for analysis as described above. Body weights as well as observation of appearance and behavior also continued throughout the study period, scored by a certified animal technician to ascertain morbidity. Body weight loss >20% is considered excessive and results in termination of the animal. Statistical comparison of mean tumor volumes between treatment groups was performed by one-way ANOVA or paired Student's *t* test, with significant difference set at $p > 0.05$.

RESULTS

Preliminary Direct Injection of Econazole into MCF-7 Xenograft Tumors in Mice

Cell-based screening assays indicated that econazole, a hydrophobic imidazole (Fig. 1) can stimulate calcium and intracellular calcium release from the endoplasmic reticulum in cancer cells thereby promoting tumor cell apoptosis. We completed preliminary *in vivo* studies to assess the anticancer effects of econazole administered as a DMSO solution via an intratumoral injection into established MCF-7 human breast tumors grown in Rag2M mice. The results of this study, summarized in Fig. 2, clearly support the idea that econazole exhibits anticancer effects. While the MCF-7 tumors treated with the DMSO vehicle control continued exponential growth, the tumors injected with econazole showed a significant tumor growth delay compared to the vehicle control group ($p = 0.007$ for 100 mg/kg dose group and $p = 0.002$ for 50 mg/kg dose group) (Fig. 2). This effect was observed at both 65 and 100 mg/kg doses, however, there was no significant difference between the two doses in tumor growth up to 64 days, suggesting that the lower dose was a reasonable target dose for ongoing efficacy studies.

Liposomal Formulation

Since intratumoral administration of DMSO-solubilized drug does not represent a viable pharmaceutical dosage form

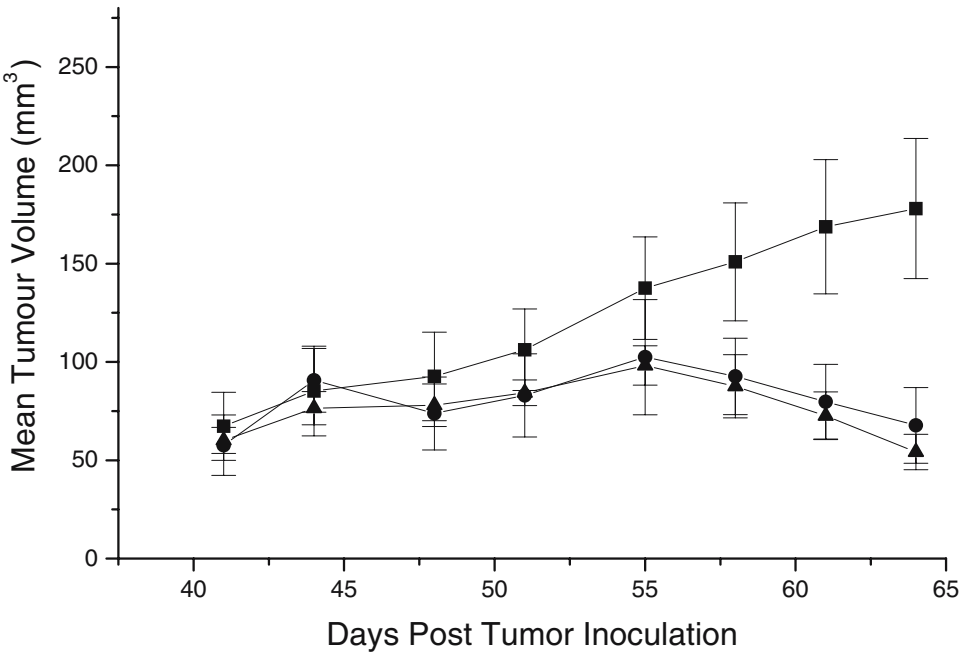

Fig. 2. Efficacy of econazole as a direct injection in MCF7 breast cancer xenografts in mice. *Squares*, DMSO vehicle control; *circles*, econazole 50 mg/ml in DMSO; *triangles*, econazole 100 mg/ml in DMSO.

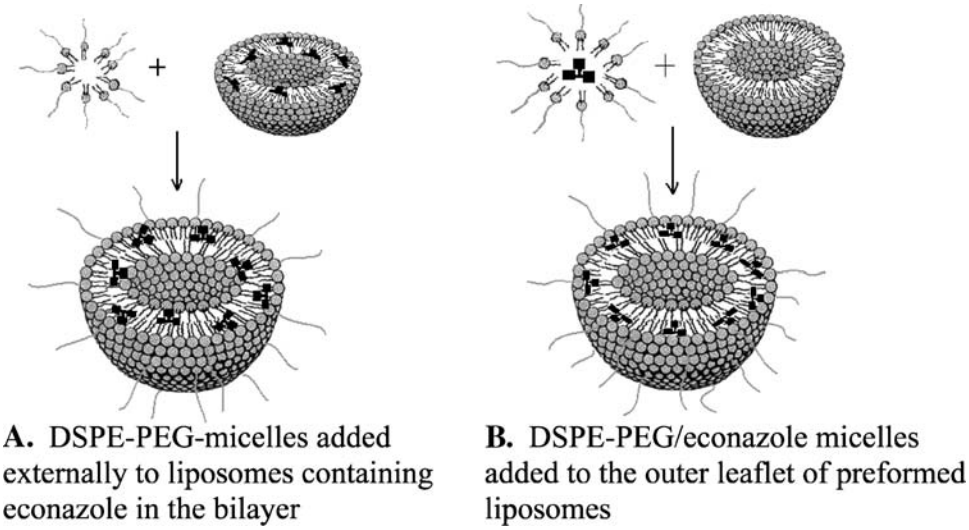

Fig. 3. A schematic representation of the formulations. DPPC or DMPC liposomes were prepared where: (A) econazole was incorporated with the lipids during the thin film stage of liposome formation, followed by hydration and extrusion, and exchange of DSPE-PEG micelles into the outer leaflet of the liposomes by passive exchange; (B) econazole was incorporated into DSPE-PEG micelles followed by passive exchange of the mixed micelles into the liposomes.

or route of administration, we pursued studies focused on the development of an econazole formulation that could be administered systemically. Two methods were considered during the early development of this project (Fig. 3). Econazole was incorporated into the lipid bilayer during liposome formation, a two step process where econazole was first incorporated into the lipid bilayer during liposome preparation, followed by a second step whereby the PEG-modified lipid DSPE-PEG_{2000} was exchanged into the preformed econazole containing liposomes (Fig. 3A, referred to as the thin film/extrusion method, TFE). In the second method, econazole was first solubilized into DSPE-PEG micelles which were subsequently mixed with preformed liposomes, a method that relied on passive equilibrium exchange of both econazole and DSPE-PEG_{2000} into the lipid bilayer (Fig. 3B, referred to as the micelle loading method, ML). The micelle-loaded formulation of econazole exhibited nearly complete drug association with the liposomes when the initial econazole to liposomal lipid ratio (*w*/*w*) was 0.05 and the concentration of econazole was 5 mg/ml (Fig. 4). Using the micelle/drug loading method described here, lipid hydration and liposome preparation occurred prior to the addition of PEG lipid, which allowed for much easier hydration and extrusion steps than typical liposomal formulation processes incorporating PEG lipid during the lipid film stage (all lipid components mixed together). It should be noted that liposomes composed of 100% DPPC formed reversible aggregates quickly after extrusion, as expected in the absence of cholesterol or PEG lipid. However, after the addition of DSPE-PEG_{2000} micelles and incubation at 50°C for 60 min with continual mixing, a stable decrease in particle

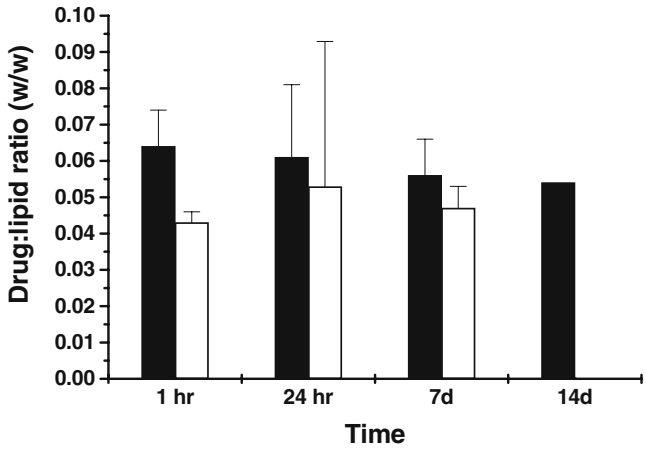

Fig. 4. Drug to lipid ratio following DSPE-PEG/econazole micelle exchange into pre-formed DPPC (*black bars*) or DMPC (*white bars*) liposomes. Data represent mean ± SD for three separate experiments within which each measurement was also performed in triplicate. Liposomes were first separated from unincorporated micelles by gel filtration on a CL4B column prior to storage and lipid and drug concentration measurements. Data at 14 days are the average of two samples.

Table I. Particle Size Analysis of Liposomes Prior to and Following DSPE-PEG or DSPE-PEG/Econazole Micelle Addition

Liposomal phospholipid formulation	Mean diameter[a]	Mean diameter after addition of DSPE-PEG micelles at 50°C (nm)
DPPC	≫1 um	152.4 ± 48.8
DPPC/econazole	143.5 ± 45.6 nm	165.3 ± 69.2
DMPC	159.4 ± 38.3 nm	160.2 ± 52.7
DMPC/econazole	141.2 ± 41.0 nm	139.9 ± 46.3

[a] Particle size determined by quasi-elastic light scattering of liposomes immediately after extrusion through 2 × 200 nm pore size filters and after the addition of DSPE-PEG micelles. Data represent mean ± SD (n = 3–6 independent preparations).

size and polydispersity indicated a reversal of the aggregation (Table I), suggesting stable PEG lipid incorporation. After the addition of DSPE-PEG_{2000} micelles with or without econazole, the mean liposomal diameter was 140–160 nm, which was not significantly different from that observed prior to addition of the micelles. The lack of a significant separate particle population <50 nm on particle size analysis was consistent with our anticipation that the DSPE-PEG micelles or DSPE-PEG/econazole micelles would be incorporated efficiently into the liposomes. When there was no PEG lipid, control DPPC/econazole liposomes [100:5 (*w*/*w*)] were not stable and coalesced within 2 h, and rapidly settled out of solution. Short term stability experiments in buffer showed that econazole remained stably associated with DPPC/DSPE-PEG (95:5 mol:mol) or DMPC/DSPE-PEG (95:5 mol:mol) liposomes in HBS (pH 7.2) at 4°C, with no significant reduction in either the drug:lipid ratio of the initial preparation over time (Fig. 4) or in particle size, with mean diameters remaining in the range of 140–160 nm throughout the study period (sizing results not shown).

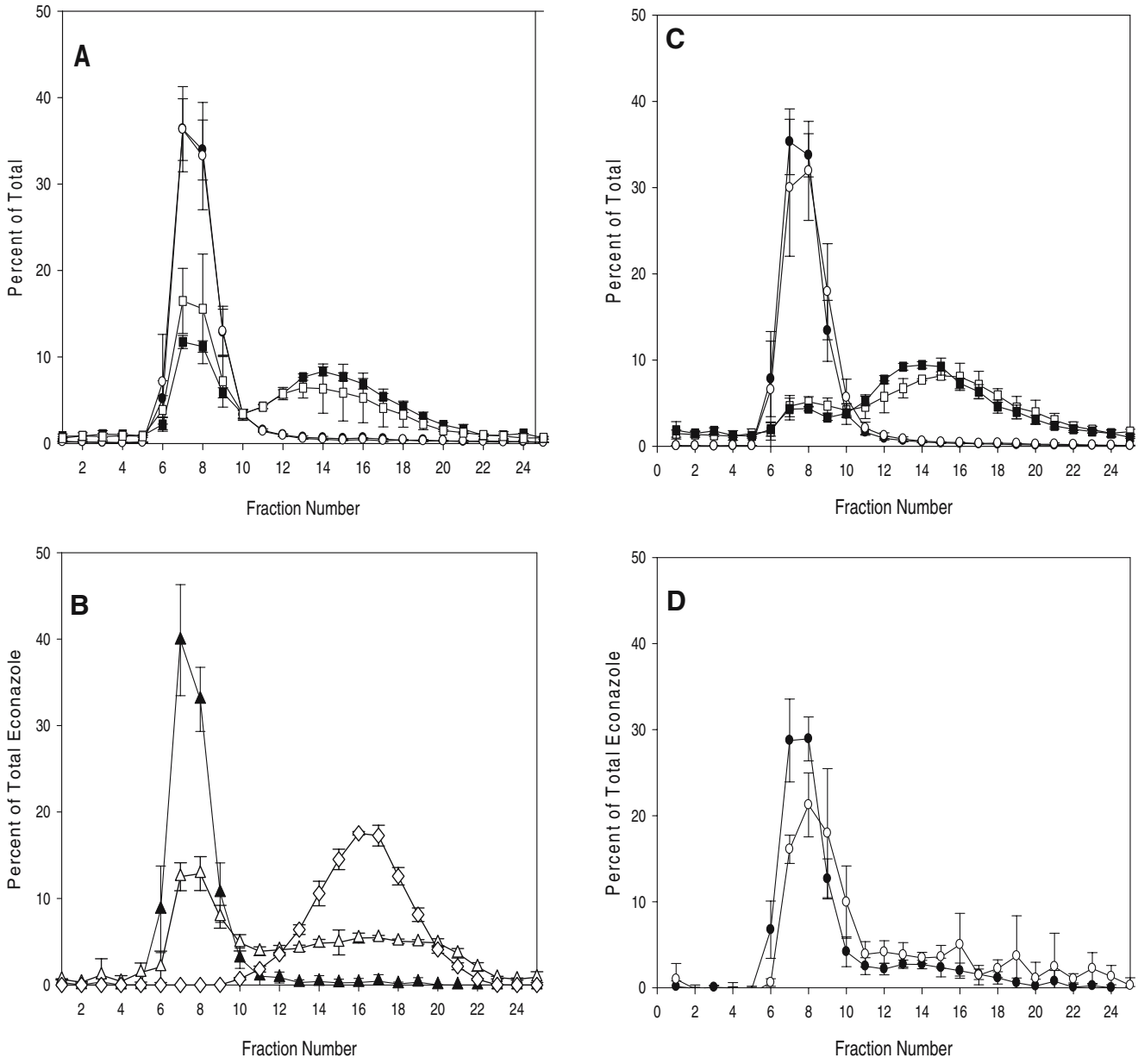

Fig. 5. Stability of micelle-loaded liposomal econazole in the presence of human plasma at 37°C. Liposomal econazole was incubated in human plasma for 30 min at 37°C, followed by fractionation by gel filtration chromatography into liposome, micelle and protein-containing fractions. (A) and (B) Liposomal econazole in DMPC/DSPE-PEG (95:5 mol:mol); (C) and (D) Liposomal econazole in DPPC/DSPE-PEG (95:5 mol:mol). Panels (A) and (C) (*top*) represent the liposomal lipid components. Panel (B) shows econazole and protein fractional distribution of DMPC/DSPE-PEG/econazole liposomes and panel (D) shows econazole fractionation from DPPC/DSPE-PEG liposomes (protein fractional distribution was same as in (B)). *Closed symbols*, samples in buffer. *Open symbols*, samples in plasma. *Circles*, liposomal lipid; *squares*, DSPE-PEG_{2000}; *triangles*, econazole; *diamonds*, total protein. Data are means ± SD (n = 3) for individual liposomal preparations.

Table II. Multidose Tolerability of Liposomal Econazole

Treatment Group	Alkaline Phosphatase (35–200 IU/l)	GGT (0–1 IU/l)	ALT (SGPT) (0–50 IU/l)	AST (SGOT) (70–900 IU/l)	Bilirubin (total) (0–7 μmol/l)	Sorbital Dehydrogenase
Empty liposomes	160 ± 10	3.7 ± 1.2 (↑) 3/3	79.3 ± 69.3	135 ± 65	5 ± 2	50.3 ± 37.3
Liposomal econazole, thin film	149 ± 12.6	3 ± 1.7 (↑) 2/3	60 ± 50.5 (↑) 1/3	188 ± 88	4.3 ± 4.5	22.7 ± 13.1
Liposomal econazole, micelle-loaded	175 ± 7	3 ± 1 (↑) 3/3	37.7 ± 6	129 ± 6.6	4 ± 2.6	26.3 ± 1.9

Serum was collected 14 days after the last of six doses (50 mg/kg) i.v. every other day. Serum was pooled from two mice to produce three samples of sufficient volume for analysis (n = 6 mice/group). Data represent mean ± SD. Arrows indicate increase (↑) above the normal range for mice, which is indicated at the top of each column in parentheses.

Plasma Stability of Liposomal Econazole *In Vitro*

For the micelle-loaded liposomal econazole preparations described above, stability in plasma was assessed by measuring drug:lipid ratio of the liposomes after incubation in plasma for 30 min at 37°C. Size exclusion chromatography was used to separate liposome-associated econazole from econazole associated with DSPE-PEG micelles or plasma proteins. For clarity, the results in Fig. 5 have been plotted in a manner that separates data from assays measuring the liposome components (panels A and C) and econazole or protein (panels B and D) of each figure. Protein fractionation was the same regardless of liposome composition. Approximately 49% of the econazole remained associated with the DPPC/DSPE-PEG liposomal fraction (Fig. 5C and D, open symbols, fractions 5–9) following the incubation period in plasma, compared to 95% for the formulations incubated in buffer (Fig. 5C and D, closed symbols, fractions 5–9). Approximately 34% of the econazole was recovered in the partially overlapping protein and micelle fractions (Fig. 5C and D, open symbols, fractions 13–19) after incubation in plasma, compared to 2% in controls incubated in buffer. In the case of DMPC/DSPE-PEG/econazole micelle-loaded liposomes, 66% was recovered in the liposomal fraction after incubation in human plasma (Fig. 5A and B, open symbols, fractions 5–9), compared to 81% in buffer (Fig. 5A and B, closed symbols, fractions 5–9), and 23% eluted in the protein/micelle fractions (Fig. 5A and B, open symbols, fractions 13–19), compared to 13% eluting in those fractions after incubation in buffer. Due to the poor solubility of econazole in HBS [<0.1 mg/ml at pH 7.2), near the limit of detection by the UV spectrophotometric assay; E. Wasan, unpublished observations] a separate free drug fraction was not detected upon elution from the column, but would likely have represented <1% of the total, based on mass balance of all collected fractions. Also important were the results showing the stability of DSPE-PEG_{2000} association with the liposomes. Approximately 37% of the DSPE-PEG was retained by the DPPC liposomal fraction (Fig. 5A, fractions 5–9) after incubation in buffer (closed squares) and 50% after incubation in plasma (open squares), whereas in the DMPC-based liposomes, only 16% was retained in the liposomal fraction (Fig. 5B, fractions 5–9) after incubation in buffer (closed squares and only 20% after incubation in plasma (open squares). As a result of this poor retention of PEG lipid by the DMPC-based formulations, DMPC liposomes were not used in the *in vivo* studies.

In Vivo Tolerability

Given the better *in vitro* stability data observed for the DPPC-based formulations, *in vivo* studies assessing the pharmacokinetic and therapeutic effects were completed using only the DPPC-based formulations. Single-dose tolerability study in Rag2M immunocompromised mice indicated that the liposomal econazole formulations, prepared using the thin film/extrusion method or the micelle loading method,

Table III. Liver Enzyme Changes in Tumor-bearing Rag2M Mice that Received Liposomal Econazole in an Efficacy Study

Treatment group	Alkaline phosphatase (35–200 IU/l)	GGT (0–1 IU/l)	ALT (SGPT) (0–50 IU/l)	AST (SGOT) (70–900 IU/l)	Bilirubin (total) (0–7 μmol/l)	Sorbital dehydrogenase
Empty liposomes	215 ± 34.6 (↑) 2/3	4.7 ± 0.6 (↑) 3/3	53 ± 11.7 (↑) 2/3	122 ± 30	5.3 ± 1.5	33 ± 4.7
Liposomal econazole, thin film	288 ± 90.7 (↑) 2/3	3.7 ± 2.3 (↑) 2/3	66 ± 7.6 (↑) 3/3	192 ± 56	6 ± 4 (↑) 1/3	32.6 ± 7.3
Liposomal econazole, micelle-loaded	225 ± 105 (↑) 1/3	3.3 ± 3.0 (↑) 2/3	36 ± 8.0	121 ± 46	8.3 ± 0.5 (↑) 1/3	25.9 ± 1.6

Serum was collected at day 59 post-tumor inoculation from mice bearing MCF-7 xenograft tumors. Treatment with liposomal econazole (50 mg/kg i.v. for six doses) occurred on days 17, 20, 22, 24, 27 and 29). Serum was pooled from two mice to produce three samples of sufficient volume for analysis (n = 6 mice/group). Data represent mean ± SD. Arrows indicate increase (↑) above normal range for mice, with the number of mice exhibiting the change indicated (e.g., two out of three samples: 2/3). Normal ranges for mice are indicated at the top of each column in parentheses.

Table IV. Serum Electrolytes and Renal Function Assessment in Tumor-bearing Rag2M Mice that Received Liposomal Econazole in an Efficacy Study

Treatment group	Na^+ (143–152 mM)	K^+ (0–1 mM)	Ca^{2+} (2.14–2.54 mM)	Phosphorus (1.73–3.51 mM)	Cl^- (103–117 mM)	CO_2 (14–28 mM)	BUN (6–17 mM)	Creatinine (30–56 μM)
Empty liposomes	148 ± 2	8.0 ± 0.3(↑ 3/3)	2.5 ± 0.03	2.5 ± 0.1	113 ± 1	27.3 ± 1.2	6.6 ± 0.5	20 ± 3
Liposomal econazole, thin filml	153 ± 5.8	7.9 ± 0.4 (↑ 3/3)	2.7 ± 0.2	2.4 ± 0.3	118 ± 5.9	27 ± 1.7	8.1 ± 3.8	23.3 ± 7.0
Liposomal econazole, micelle-loaded	150 ± 1.5	8.3 ± 0.5 (↑ 2/3)	2.6 ± 0.1	2.5 ± 0.1	115 ± 1.5	28.3 ± 1.5	6.1 ± 0.4	22.7 ± 5.0

Serum was collected at day 59 post-tumor inoculation from mice bearing MCF-7 xenograft tumors. Treatment with liposomal econazole (50 mg/kg i.v. for six doses) occurred on days 17, 20, 22, 24, 27 and 29). Serum was pooled from two mice to produce three samples of sufficient volume for analysis (n = 6 mice/group). Data represent mean ± SD. Arrows indicate increase (↑) above normal range for mice, which is indicated at the top of each column in parentheses.

were well tolerated when administered intravenously at an econazole dose of 50 mg/kg [drug:lipid ratio = 0.05 (*w*/*w*)] (data not shown). The multidose tolerability study (i.v., Q2D × 6) also suggested that the DPPC-based liposomal econazole formulations were well tolerated at 50 mg/kg econazole. Serum was collected 14 days after the last of six doses and as part of the efficacy study (see below) at day 59 post-tumor inoculation (42 days after treatment stopped). As a class, the imidazoles have been associated with hepatotoxicity, therefore serum was analyzed for elevations in liver enzymes (alkaline phosphatase, ALT, AST, GGT, bilirubin and sorbital dehydrogenase) and other serum chemistry parameters, as summarized in Tables II, III and IV.

In the multidose tolerability assessment, serum analysis indicated mild elevations in liver enzymes (ALT, GGT) in the liposomal econazole groups and less so in the vehicle control group (n = 3 mice/group, lipid dose in all groups 1,000 mg/kg) compared to the laboratory normal ranges for mice (Table II). Table III summarizes the results of serum analysis from the efficacy study, where increases in alkaline phosphatase, AST and GGT were observed. Alkaline phosphatase was elevated in all groups receiving liposomes, and in groups receiving econazole, bilirubin was slightly elevated in one of three samples in both groups. Results of serum electrolyte analysis showed potassium levels above the normal range for mice in all groups receiving liposomes, however, BUN and creatinine were not elevated (Table IV). The reason for this isolated electrolyte imbalance is unknown. Necropsy revealed pale liver and kidneys in several animals in all groups of the multidose study and the efficacy study, including the vehicle control group, which is consistent with effects associated with the relatively high lipid dose (21). However, it should be noted that these studies were completed in tumor bearing animals and these elevations could be secondary to the cancer burden in these animals. Formal toxicology studies need to be completed in order to establish the safety of the current formulation. Nevertheless, econazole-containing liposomes were no more toxic than empty liposomes, and all formulations were well tolerated at the doses used.

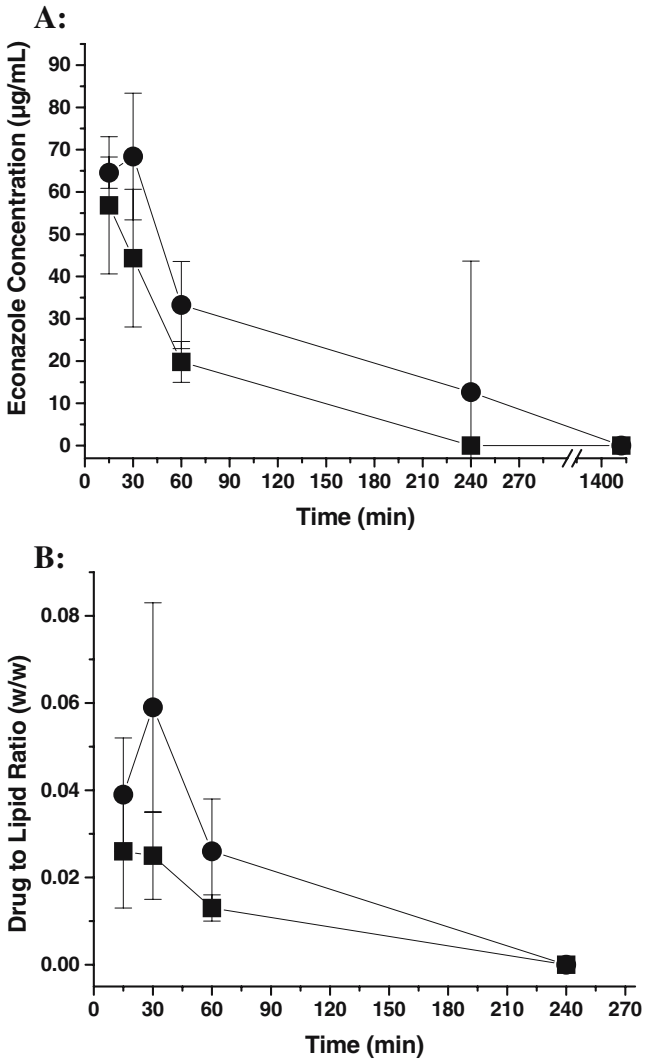

Fig. 6. Plasma elimination profile of liposomal econazole. Points represent six mice per timepoint (mean ± SD). *Squares*, liposomal econazole prepared by the thin film/extrusion method; *circles*, liposomal econazole prepared by the micelle loading method. (A) Econazole elimination from plasma. (B) Drug to lipid ratio (*w*/*w*) *vs* time.

Pharmacokinetics of Liposomal Econazole

Rag2M mice were injected intravenously with liposomal econazole that was prepared by either the thin film/extrusion

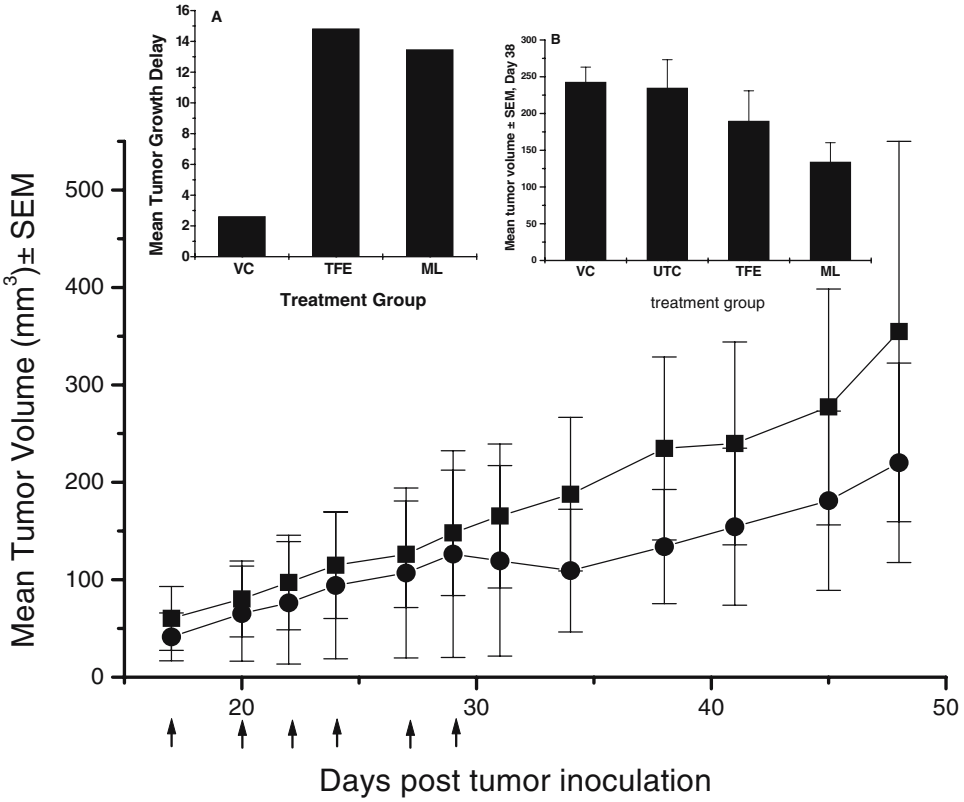

Fig. 7. Efficacy of liposomal econazole against MCF-7 tumors grown as xenografts in immunocompromised Rag2M mice. Treatment was with liposomal econazole composed of DPPC/DSPE-PEG (95:5 mol:mol). *Squares*, empty liposome vehicle control; *circles*, liposomal econazole prepared by the micelle loading method. Treatment was on days 17, 20, 22, 24, 27 and 29 (indicated as ↑ on graph), starting when tumors were approximately 50 mm^3. Data represent mean ± SEM ($n = 6$). *Inset A*, tumor growth delay to reach 300 mm^3 compared to untreated controls, as absolute difference of means. *VC*, empty liposome control [DPPC/DSPE-PEG (95:5 mol:mol)]; *TFE*, liposomal econazole prepared by the thin film/extrusion method; *ML*, liposomal econazole prepared by the micelle loading method. *Inset B*, tumor volume at day 38 post tumor inoculation (mean ± SD, n=6).

method or by the micelle loading method. Analysis of econazole concentration in the plasma *vs* time (Fig. 6) showed that econazole was cleared from the plasma by 2 h and that elimination appears to follow a first-order elimination process. The area under the curve for the measured timepoints ($AUC_{0–240\ min}$) was estimated to be 196 μg/ml min for the thin film/extrusion liposomal econazole and 281 μg/ml min for the micelle-loaded liposomal econazole and plasma half-life of approximately 30.9 and 34.3 min, respectively. The drug-to-lipid ratio, a measure of how well the econazole was retained by the liposomes following administration, was significantly different between the two formulations at 15 min ($p = 0.036$) and 60 min ($p = 0.012$), with the micelle-loaded form showing a higher drug-to-lipid ratio at those timepoints.

Efficacy of Liposomal Econazole in MCF-7 Xenografts in Rag2M Mice

Efficacy studies were completed in Rag2M mice bearing tumors arising following subcutaneous injection of human MCF-7 breast cancer cells. This is an estrogen receptor positive cell line, therefore tumor growth was supported by a 60-day slow-release estrogen pellet was implanted subcutaneously in the mice 1 day prior to tumor cell inoculation. Tumor-bearing mice received empty liposomes (vehicle control), liposomal econazole prepared by passive loading (thin film extrusion method, TFE) or micelle-loaded liposomal econazole (ML) every other day for six doses. Tumor measurements were performed for 60 days, and the effect of treatment on tumor growth is shown in Fig. 7. For animals that were left untreated, a tumor volume of 300 mm^3 was observed 44 days after tumor cell implantation, whereas there was only a 2.6-day tumor growth delay in animals treated with empty liposomes, but a 14-day delay for both liposomal econazole groups in the mean number of days to reach 300 mm^3 (i.e., animals treated with liposomal econazole had tumors that did not reach 300 mm^3 until a mean of 58 days) (Fig. 7A inset). Thus, although liposomal econazole did not cause tumor regression, its administration was associated with a trend to a delay in tumor growth, with the most difference in tumor size observed around day 38 (Fig. 7B inset), although

tumor size did not reach statistical significance due to the variability in tumor size within each group.

DISCUSSION

Previous attempts in our laboratory to prepare liposomal econazole were limited by its poor water solubility. The use of PEG lipid and ethanol as a solubilizing agent in this study improved drug loading of econazole into preformed phospholipid liposomes composed of DPPC or DMPC. Solubilization of econazole in the micelles, and possibly other hydrophobic agents, allows greater flexibility in designing parenteral dosage forms for *in vivo* studies. It should also be noted that when DSPE-PEG_{2000} is incorporated into DPPC or DPMC liposomes during manufacturing, the extrusion process can be difficult and laborious due to their foamy and viscous nature, which can also lead to loss of materials. This was avoided by adding the PEG lipid externally and allowing it to exchange into the DPPC or DMPC liposomes. We have demonstrated that incorporation of PEG lipid and econazole (5 mg/ml) can be achieved efficiently by this method. Adding PEG lipid externally likely generates asymmetric liposomes such that most or all of the PEG lipid will be associated with the outer bilayer leaflet, based on the slow rate of intermembrane flipping of this molecule (22). Ethanol was used here as a cosolvent for econazole, but it may also be functioning as a membrane permeation enhancer by reducing the interfacial tension at the liposomal membrane (23–26). Like other imidazole antifungal drugs, econazole likely associates with the liposomal membrane between lipid molecules (27) upon exchange into the pre-formed liposomes, although the mechanism of drug–lipid association is presently still under study in our laboratory.

In vivo, the pharmacokinetic profile of liposomal econazole shows that detectable drug levels were achieved in the plasma for at least 2 h following i.v. administration of econazole formulated using either the thin film/extrusion method or the micelle loading method. Pandy *et al.*, recently showed that econazole administered to mice intravenously in 25% methanol is completely cleared from the plasma by 1 h (26). It was anticipated that the micelle-loaded liposomes would retain less drug in circulation than those where econazole was loaded by the thin film/extrusion method into both leaflets of the bilayer. The *in vivo* results suggest, however, that the micelle-loaded liposomes retained econazole better. This result may be reflective of a higher concentration of PEG lipid on the exterior of the micelle-loaded liposomes than the conventional liposomes at the same total lipid:PEG lipid molar ratio, thereby improving liposome stability in the bloodstream (28). Furthermore, the econazole may be partitioning more effectively into the DSPE part of DSPE-PEG rather than DMPC or DPPC of the liposomes. This may explain in part the better econazole retention of the micelle-loaded liposomes where the outer leaflet theoretically has twice the DSPE-PEG concentration as the liposomes prepared conventionally (if we ignore PEG lipid packing constraints in the conventional liposomes).

Efficacy studies in MCF-7 xenograft tumors in mice showed a dramatic growth inhibition following direct injection of econazole in DMSO into the tumors (Fig. 2), and a slight delay in tumor growth during dosing with the i.v. liposomal econazole preparations described here (Fig. 7). The dose used in these studies (50 mg/kg) did not cause significant toxicity as judged by single and multidose studies, including body weight loss, qualitative assessments of toxicity, and serum chemistry analysis, e.g., liver enzymes. Although efficacy results suggested that econazole in DMSO (notably, a permeability enhancer) was more effective in this tumor model, use of intratumoral injections and DMSO solubilized drug would not be appropriate when considering later clinical development, and an alternative, less toxic formulation that could be administered i.v. was successfully achieved here. It should be noted that the full mechanism of action of econazole is still under investigation, and that ongoing studies are employing other tumor models as well to determine the range of application of econazole as an anticancer agent. While there was no statistical difference in treatment efficacy (e.g., tumor growth delay or tumor size at a given timepoint) comparing the thin film/extrusion method and micelle loading method in spite of a difference in AUCs measured in the pharmacokinetic study, it may be that the size of the study was too small to detect a small difference of treatment effect with the existing variation in tumor size within each group. The trend to improved activity with micelle-loaded liposomal econazole is seen in Fig. 7B inset, where tumors in mice receiving that treatment were slightly smaller around day 38.

The results obtained with the econazole formulation prepared using the micelle loading method are promising. Work is ongoing to improve the drug concentration in the liposomes. We are also in the process of evaluating the influence of dose intensity (number of treatments, dosing interval) and treatment schedule on efficacy in several tumor models. Pharmacokinetic studies will be expanded to include analysis of drug levels in tumors, and we will use these results in establishing the mechanism of drug delivery and activity in the *in vivo* setting.

CONCLUSIONS

Econazole is an anticancer agent with great potential for breast and prostate cancer, osteosarcoma and leukemia, which may be particularly suitable for combination therapy with other drugs based on its unique mechanism of action. Other advantages include the lack of susceptibility to major drug resistance mechanisms and its excellent tolerability. A parenteral formulation of econazole was achieved using phospholipid liposomes containing PEG lipid. The addition of PEG lipid micelles containing a drug substance to promote drug exchange into pre-formed liposomes is a novel technique. This formulation is presently under development for the treatment of solid tumors, including breast cancer. It is hoped that this method of drug incorporation into liposomes will also be applicable to other hydrophobic drugs.

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