ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1998, p. 45–52 Vol. 42, No. 1
0066-4804/98/$04.00+0

Antibacterial Efficacy against an In Vivo *Salmonella typhimurium* Infection Model and Pharmacokinetics of a Liposomal Ciprofloxacin Formulation

MURRAY S. WEBB,[1]* NANCY L. BOMAN,[1] DAVID J. WISEMAN,[1] DAWN SAXON,[1] KYM SUTTON,[1] KIM F. WONG,[2] PATRICIA LOGAN,[1] AND MICHAEL J. HOPE[1,3]

Inex Pharmaceuticals Corporation, Burnaby, British Columbia, Canada V5J 5J8[1]; Liposome Research Unit, Department of Biochemistry, University of British Columbia Vancouver, British Columbia, Canada V6T 4E6[2]; and Skin Barrier Research Laboratory, Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada V6T 2B5[3]

Received 7 May 1997/Returned for modification 10 October 1997/Accepted 27 October 1997

The fluoroquinolone antibiotic ciprofloxacin has been encapsulated into large unilamellar vesicles (LUV) at efficiencies approaching 100%. Drug accumulation proceeded in response to a transmembrane gradient of methylammonium sulfate and occurred concomitantly with the efflux of methylamine. A mechanism for the encapsulation process is described. LUV composed of dipalmitoylphosphatidylcholine-cholesterol (DPPC/chol), distearoylphosphatidylcholine-cholesterol (DSPC/chol), or sphingomyelin-cholesterol (SM/chol) increased the circulation lifetime of ciprofloxacin after intravenous (i.v.) administration by >15-fold. The retention of ciprofloxacin in liposomes in the circulation decreased in the sequence SM/chol > DSPC/chol > DPPC/chol. Increased circulation lifetimes were associated with enhanced delivery of the drug to the livers, spleens, kidneys, and lungs of mice. Encapsulation of ciprofloxacin also conferred significant increases in the longevity of the drug in the plasma after intraperitoneal administration and in the lungs after intratracheal administration in comparison to free ciprofloxacin. The efficacy of a single i.v. administration of an SM/chol formulation of ciprofloxacin was measured in a *Salmonella typhimurium* infection model. At 20 mg of ciprofloxacin per kg of body weight, the encapsulated formulation resulted in 10^3- to 10^4-fold fewer viable bacteria in the livers and spleens of infected mice than was observed for animals treated with free ciprofloxacin. These results show the utility of liposomal encapsulation of ciprofloxacin in improving the pharmacokinetics, biodistribution, and antibacterial efficacy of the antibiotic. In addition, these formulations are well suited for i.v., intraperitoneal, and intratracheal or aerosol administration.

Ciprofloxacin is a synthetic bactericidal fluoroquinolone antibiotic which inhibits the activity of bacterial DNA gyrase, resulting in the degradation of bacterial DNA by exonuclease activity. Consequently, ciprofloxacin has broad-spectrum efficacy against a wide variety of bacteria, including *Staphylococcus aureus*, streptococci, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, and *Mycobacterium avium* complex (9, 11, 42). For example, a comprehensive study showed excellent in vitro ciprofloxacin activity against >20,000 clinical isolates of members of the family *Enterobacteriaceae*, non-*Enterobacteriaceae* gram-negative bacteria, gram-positive bacteria, anaerobic bacteria, and other types of bacteria (37). Despite the enormous success with ciprofloxacin, there are some factors which limit the drug's clinical utility, such as its poor solubility at physiological pH, bitter taste in solution, and rapid renal clearance. For example, in order to administer a typical ≈0.5-g intravenous (i.v.) dose, the drug must first be diluted to <2 mg/ml and infused slowly to avoid precipitation at the site of injection.

Encapsulation of therapeutic agents in liposomal carriers is known to be an effective method for reducing drug toxicity, for increasing the circulation longevity of drugs after parenteral administration, and for increasing the accumulation of drugs at sites of disease. In particular, liposomal encapsulation has been shown to improve the therapeutic index of anticancer agents, such as doxorubicin (23, 46) and vincristine (4, 24, 26, 27, 46, 48), as well as antibiotics, such as gentamicin (1, 17, 31, 33), streptomycin (7, 47), vancomycin (36), amikacin (1, 8, 35), cefoxitin (18), and ofloxacin (10). One explanation for the enhanced efficacy observed for encapsulated antibiotics in animal models is the natural targeting of lipid carriers to the fixed macrophages of the reticuloendothelial system, which often harbor microorganisms and protect them from free drug (18).

Ciprofloxacin passively encapsulated in multilamellar vesicles (MLV) has shown enhanced in vitro efficacy against *Mycobacterium avium-Mycobacterium intracellulare* complex infection in human peripheral blood mononuclear cells (22). Similarly, ciprofloxacin loaded into unilamellar liposomes has shown enhanced in vitro activity against a *Mycobacterium avium* infection of murine J774 macrophages (34). Moreover, several reports have demonstrated enhanced in vivo efficacy against *Francisella tularensis*, *Brucella melitensis*, and *Salmonella dublin* infections in mice with ciprofloxacin passively encapsulated in MLV (6, 21, 49). It should be noted, however, that the drug pharmacokinetics, accumulation at infection site, and antibacterial efficacy will be significantly altered both by the liposome size (16, 50) and by the rate of drug leakage. In general, a drug that is actively retained inside a carrier by a transmembrane ion gradient leaks more slowly than a drug that has been passively encapsulated (5, 15).

This paper describes and characterizes the active loading of ciprofloxacin into three different unilamellar liposomal carriers with a transmembrane gradient of methylammonium sulfate.

* Corresponding author. Mailing address: Inex Pharmaceuticals Corporation, 100-8900 Glenlyon Parkway, Burnaby, British Columbia, Canada V5J 5J8. Phone: (604) 264-9954. Fax: (604) 264-9690. E-mail: mwebb@inexpharm.com.

Previously, we have shown that ciprofloxacin could not be encapsulated by a simple pH gradient technique similar to that employed to load doxorubicin (29) or vincristine (24), but was accumulated when an ammonium sulfate transmembrane gradient was applied. For this study, a gradient of methylammonium sulfate was used to create the proton gradient responsible for drug accumulation. Other ammonium salts can be employed as long as the neutral amine formed after complete ionization can diffuse out of the vesicle (see Discussion for details concerning the mechanism of drug loading). In addition, we have evaluated the effect of liposomal encapsulation on drug pharmacokinetics after i.v., intraperitoneal (i.p.), and intratracheal administration and describe the effects of encapsulation on the antibacterial efficacy of ciprofloxacin against an intracellular *Salmonella typhimurium* infection in mice.

MATERIALS AND METHODS

Materials. Egg sphingomyelin (SM) was obtained from Avanti Polar Lipids (Alabaster, Ala.), dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) were obtained from Northern Lipids (Vancouver, British Columbia, Canada), and cholesterol was obtained from Sigma Chemical Company. Ciprofloxacin hydrochloride and [^{14}C]ciprofloxacin were kindly supplied by Bayer. [^{14}C]methylammonium sulfate was synthesized by the method described below for the synthesis of methylammonium sulfate, but with [^{14}C] methylamine obtained from Amersham. Tritiated cholesterylhexadecyl ether (^{3}H-CHE) was also obtained from Amersham. Methylammonium chloride was obtained from Sigma Chemical Company, and methylamine was obtained from BDH (Vancouver, British Columbia, Canada). The animals used in this study were 6- to 8-week-old female ICR and BALB/c mice, both obtained from Charles River Laboratories. *Staphylococcus aureus* RN450 was obtained from J. Davies, Department of Microbiology and Immunology, University of British Columbia. *Salmonella typhimurium* SL1344 was obtained from B. Finlay, Department of Biochemistry & Molecular Biology and Department of Microbiology & Immunology, University of British Columbia, Vancouver, British Columbia, Canada.

Methods. (i) Ciprofloxacin assay. Liposomal ciprofloxacin, in samples not containing [^{14}C]ciprofloxacin, was assayed by alkalinization of the sample to pH 12 with NaOH and then extraction from the lipid by a two-phase Bligh and Dyer extraction procedure (2). The aqueous upper phase was removed after centrifugation and was assayed for A_{275} and compared to an aqueous upper-phase blank. This procedure recovered >95% of ciprofloxacin present in the samples and was linear in the range between 0 and at least 65 μM ciprofloxacin (r^2 for this regression was 0.9998).

(ii) Synthesis of methylammonium sulfate. Methylammonium sulfate was prepared from methylamine (40% [wt/vol]in H_2O), and concentrated sulfuric acid was prepared by the dropwise addition of 34.5 ml of concentrated sulfuric acid to 100 ml of methylamine solution with continuous stirring in the cold (ice bath). The pH of the final solution was adjusted to 6.0 to 7.0 with dilute sulfuric acid or dilute methylamine. The methylammonium sulfate was dried by rotary evaporation at 80°C. The methylammonium sulfate slurry was resuspended with 200 ml of absolute ethanol and then dried and resuspended once with 200 ml and twice with 100 ml of ethanol. The resulting methylammonium sulfate slurry was taken up with 100 ml of anhydrous ether, filtered, and then dried extensively under vacuum. Confirmation of chemical identity and purity was performed by comparison with methylammonium chloride by ^{13}C-nuclear magnetic resonance (^{13}C-NMR).

(iii) Formulation of ciprofloxacin into liposomes. Liposomes were prepared by dissolving a total of 100 mg of lipid comprised of phospholipids (DPPC, DSPC, or SM) and cholesterol (DPPC/chol, DSPC/chol, and SM/chol, respectively) at phospholipid/cholesterol molar ratios of 55/45 in $CHCl_3$ or a mixture of $CHCl_3$ and CH_3OH. Bulk solvent was removed under a stream of nitrogen gas, and then trace solvent was removed by holding the lipid film under high vacuum overnight. The lipid films were hydrated by the addition of 1.0 ml of 0.3 M methylammonium sulfate and then vortexed and subjected to brief heating (50°C for DPPC/chol, 65°C for DSPC/chol and SM/chol) to produce MLV. The MLV suspensions were subjected to five freeze-thaw cycles between −196°C and the temperatures described above. Large unilamellar vesicles (LUV) were produced by 10 passages of the MLV suspensions through two stacked 0.1-μm-pore-diameter filters with an extruder (Lipex Biomembranes, Vancouver, British Columbia, Canada) maintained at 65°C as characterized previously (14, 32). Vesicle diameters were confirmed by quasielastic light scattering with a Nicomp model 270 submicron particle sizer.

Ciprofloxacin was loaded into liposomes in response to a transmembrane gradient of the methylammonium that was established by overnight dialysis of liposomes against 1,000 volumes of 150 mM NaCl. Ciprofloxacin loading was initiated by the addition of the liposomal suspension to the appropriate quantity of the ciprofloxacin ($HCl \cdot H_2O$), either as a dry powder or as a 25-mg/ml solution (in H_2O), so as to achieve a final ciprofloxacin/lipid molar ratio of 0.25 or 0.3. Loading was allowed to proceed for 60 min at 50°C (DPPC/chol) or 65°C (DSPC/chol and SM/chol).

(iv) Measurement of ciprofloxacin loading and transmembrane pH gradient. The encapsulation of ciprofloxacin into liposomes was assessed by column chromatography. At various times during the uptake of ciprofloxacin into liposomes, aliquots were diluted in saline, and then 50 or 100 μl was loaded onto 1-ml columns of Sephadex G-50 that had been equilibrated in saline and precentrifuged before use (38). The loaded columns were then centrifuged for 1 to 1.5 min at 2,000 rpm, and the eluate, containing the void volume, was recovered for analysis of lipid (liquid scintillation counting [LSC]) and ciprofloxacin (LSC or A_{275}).

The transmembrane pH gradient across the liposome membrane during the uptake of ciprofloxacin was determined with [^{14}C]methylamine as characterized previously (12, 41). Liposomes of DPPC/chol (labeled with ^{3}H-CHE) were equilibrated in the presence of 1 μCi of [^{14}C]methylamine. Ciprofloxacin was added to the sample, which was then heated to 60°C. At various times, the external pH was determined with a pH probe, and the transmembrane pH gradient and ciprofloxacin loading were determined by passing the samples over the 1-ml Sephadex G-50 columns as described above. The column eluates were analyzed for [^{14}C]methylamine by LSC and for ciprofloxacin by A_{275}. The intraliposomal pH was calculated assuming a liposome trap volume of 1.15 μl/μmol of lipid (32).

(v) Pharmacokinetics and biodistribution of ciprofloxacin. Ciprofloxacin (labeled with [^{14}C]ciprofloxacin at 0.5 μCi/mg of ciprofloxacin) was encapsulated in liposomes in response to a methylammonium sulfate gradient as described above. The liposomes were composed of DPPC/chol, DSPC/chol, or SM/chol (55/45 [mol/mol]) labeled at 20 μCi/100 mg of lipid with ^{3}H-CHE (a nonexchangable and nonmetabolizable lipid radioactive tracer [43]). Pharmacokinetic studies were performed by injecting liposomal ciprofloxacin formulations or free ciprofloxacin into ICR mice via the lateral tail vein at a dose of 15 mg of ciprofloxacin/kg of body weight. In other experiments, free ciprofloxacin and liposomal ciprofloxacin were administered by i.p. injection or by intratracheal instillation after halothane anesthetization. At various time points after administration, the mice were anesthetized, and blood was recovered via cardiac puncture. Subsequently, the animals were terminated by cervical dislocation, and the liver, spleen, lung, kidney, and leg muscle were recovered. Plasma and tissue homogenates were assayed for ciprofloxacin and lipid by LSC. Concentrations of lipid and ciprofloxacin in tissue were corrected for the contribution from the blood.

(vi) Determination of in vivo efficacy versus *Salmonella typhimurium*. *Salmonella typhimurium* SL1344 was grown overnight in Luria-Bertani broth and then centrifuged and washed in phosphate-buffered saline (PBS) and subsequently diluted to achieve a final suspension containing 1,000 CFU/ml. Female BALB/c mice (6 to 8 weeks old) were injected i.v. into the tail vein with inocula containing 60 to 90 CFU per mouse.

Twenty-four hours after infection, the mice were treated with free ciprofloxacin (in saline) or liposomal ciprofloxacin by i.v. administration at doses of 1 or 20 mg of ciprofloxacin/kg. The solutions of both free and liposomal ciprofloxacin were sterilized by passage through a 0.2-μm-pore-diameter filter prior to administration. The liposomal ciprofloxacin formulations used in these experiments were 130-nm SM/chol (55/45 [mol/mol]) liposomes containing ciprofloxacin at a ciprofloxacin/lipid molar ratio of 0.239. (For these experiments, the ciprofloxacin was loaded at a drug/lipid ratio of 0.25, compared to 0.30, to improve drug retention [30].) At 4 or 5 days postinfection, the mice were sacrificed, and the spleens and livers were removed to sterile 6-ml tubes on ice. Individual organs were transferred to sterile plastic Stomacher bags (Seward Medical, London, England), and the organs were crushed and homogenized for 90 s in the presence of PBS with the Stomacher apparatus. Aliquots (100 μl) of the organ suspensions were serially diluted in PBS to a maximum of 10^5-fold dilution and then were plated on duplicate MacConkey agar plates. Plates were incubated overnight at 37°C, and the resulting whitish, light-red (Lac^-) colonies were counted for plates containing between 30 and 300 colonies.

RESULTS

Characterization of ciprofloxacin loading. The effect of solution pH, in the range between 4 and 11, on the solubility of aqueous ciprofloxacin is shown in Fig. 1. At pH values below $pK_1 = 6.0$ and above $pK_2 = 8.8$, ciprofloxacin has a net charge and is highly soluble. However, in the pH range between these pK values, the compound is zwitterionic or neutral and is practically insoluble (Fig. 1). Throughout this study, we employed ciprofloxacin hydrochloride. Dissolved in water at a concentration of 25 mg/ml, this solution has a pH of 3.5.

The kinetics of ciprofloxacin uptake into LUV in response to a transmembrane gradient of methylammonium sulfate are shown in Fig. 2A. The amount of drug encapsulated and retained inside the vesicles was determined directly from the ciprofloxacin/lipid ratio (20, 25). Using 120-nm LUV and an

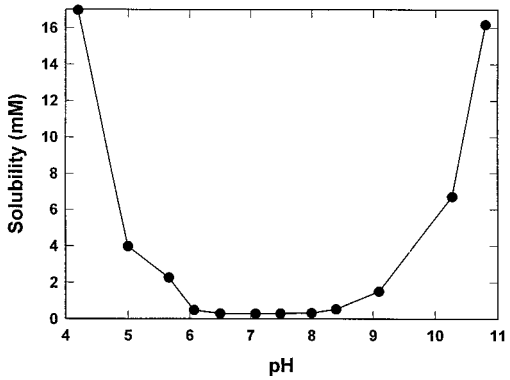

FIG. 1. Solubility of ciprofloxacin in aqueous solutions buffered in the pH range between 4 and 11. Ciprofloxacin hydrochloride was dissolved to saturation in 50 mM HEPES at various pHs. Excess drug was removed by centrifugation, and the supernatant was assayed for ciprofloxacin content by A_{275}.

initial ciprofloxacin/lipid molar ratio of approximately 0.3, typical entrapment efficiencies of 95 to 100% were observed in formulations comprised of DPPC/chol, DSPC/chol, and SM/chol (data not shown). Complete uptake of ciprofloxacin within 1 h required that the loading was performed at temperatures above the L_α-to-L_β lipid phase transition temperature for the phospholipid employed (i.e., at 50°C for DPPC/chol and 65°C for DSPC/chol and SM/chol), despite the presence of sufficient cholesterol to eliminate the L_α-to-L_β lipid phase

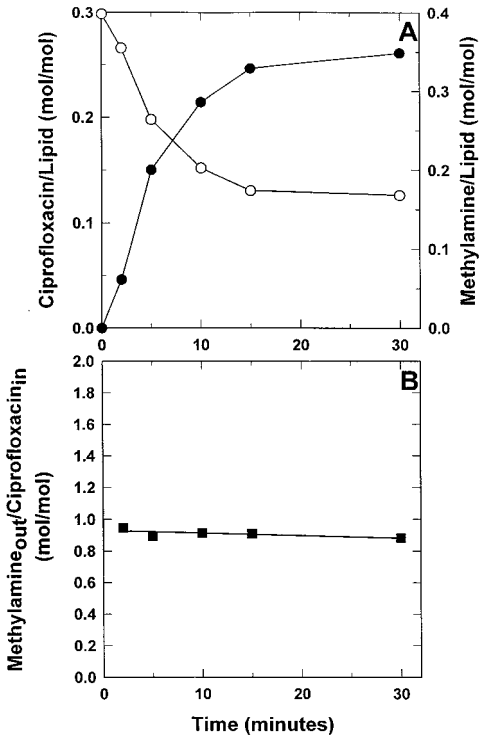

FIG. 2. Loading of ciprofloxacin into liposomes. (A) Uptake of ciprofloxacin, expressed as the ciprofloxacin/lipid ratio (●) and release of [^{14}C]methylamine, expressed as the methylamine/lipid ratio (○). Ciprofloxacin uptake was initiated by the addition of ciprofloxacin to 100-nm unilamellar liposomes composed of DPPC/chol (55/45 [mol/mol]) and possessing a transmembrane 0.3 M methylammonium sulfate gradient. (B) Calculated stoichiometry of moles of methylamine released/moles of ciprofloxacin loaded.

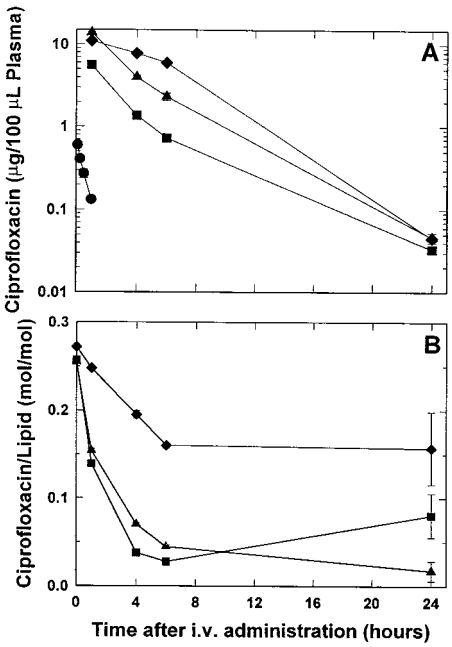

FIG. 3. Pharmacokinetics of free and liposomal ciprofloxacin after i.v. administration. (A) Concentrations of ciprofloxacin in plasma after i.v. administration of free ciprofloxacin (●) or ciprofloxacin encapsulated in liposomes comprised of DPPC/chol (■), DSPC/chol (▲), or SM/chol (◆). The dose of ciprofloxacin injected for all treatments was 15 mg/kg. (B) Ciprofloxacin/lipid ratios in plasma after i.v. administration of the DPPC/chol (■), DSPC/chol (▲), or SM/chol (◆) formulation of liposomal ciprofloxacin. Data represent means ± standard errors from three mice.

transition. DPPC/chol LUV possessing a transmembrane gradient of methylammonium sulfate retained >96% of the encapsulated methylamine within the LUV during 42 h of dialysis at 21°C (data not shown). However, upon the addition of external ciprofloxacin and subsequent ciprofloxacin loading, methylamine rapidly effluxed from the LUV (Fig. 2A). The molar stoichiometry of methylamine to ciprofloxacin varied in the range between 0.88 and 0.95 during drug accumulation (Fig. 2B), suggesting that a one-to-one exchange of ciprofloxacin for methylamine occurred during drug loading. Analysis of ciprofloxacin uptake and the simultaneous changes of the internal and external pH of 100-nm DPPC/Chol LUV showed that the internal vesicle pH increased from approximately 2.8 prior to the addition of drug to the external solution to approximately 3.1 during the encapsulation process (data not shown). These results are consistent with the data (Fig. 2A) showing that a significant amount of methylamine remained inside the vesicles after ciprofloxacin loading. This is sufficient to maintain the internal pH and retain the antibiotic (see Discussion). It should be noted that all three liposomal ciprofloxacin formulations retained 100% of the encapsulated drug during storage for 18 weeks at 4°C, 12 weeks at 21°C, or 8 weeks at 37°C (data not shown).

In vivo pharmacokinetics and biodistribution of free and liposomal ciprofloxacin. After i.v. administration, free ciprofloxacin was removed from the circulation of mice with a half-life of approximately 0.2 h (Fig. 3A). Encapsulation of ciprofloxacin in all of the carriers increased the circulation half-life of ciprofloxacin from 0.2 h to >3 h (Fig. 3A), representing 43- to 105-fold increases in concentrations of ciprofloxacin in plasma occurring as a consequence of encapsulation. For example, at 1 h after i.v. administration, the ciprofloxacin con-

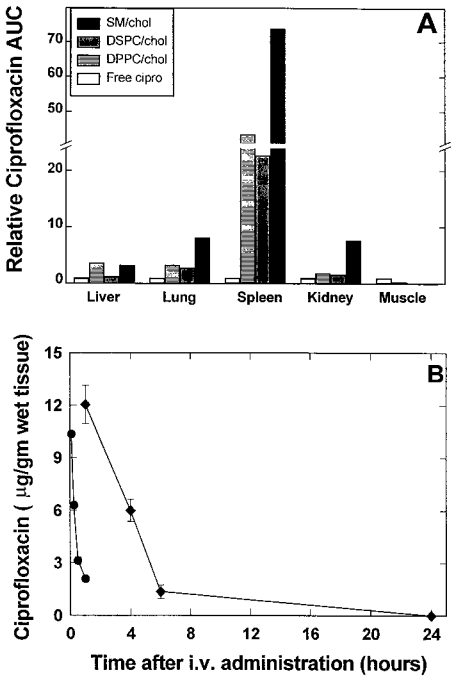

FIG. 4. Summary of the biodistribution of free and liposomal ciprofloxacin (cipro) after i.v. administration. Area under the concentration-time curve (AUC) values over 24 h for the different liposomal formulations are expressed relative to that for free ciprofloxacin in liver, lung, spleen, kidney, and muscle (A). Ciprofloxacin concentrations in the lungs of mice after i.v. administration of free ciprofloxacin (●) or ciprofloxacin encapsulated in SM/chol liposomes (◆) are shown as an example (B). The dose of ciprofloxacin injected for all treatments was 15 mg/kg. Data represent means ± standard errors from three mice.

centrations were 0.132 μg/100 μl of plasma for free ciprofloxacin and between 5.62 and 13.8 μg/100 μl of plasma for the liposomal ciprofloxacin formulations. Ciprofloxacin that leaked from the liposomes would be expected to be removed from the circulation at rates identical to that for free drug administered i.v. (Fig. 3A) (28). Higher levels of ciprofloxacin in plasma were observed in the SM/chol formulation and were a consequence of the higher ciprofloxacin/lipid ratio in the plasma at various times after i.v. administration in this formulation compared to those of the DPPC/chol and DSPC/chol formulations (Fig. 3B). The SM/chol carrier retained significantly greater proportions of the encapsulated ciprofloxacin than either the DSPC/chol or DPPC/chol carriers. For example, at 4 and 6 h after i.v. administration of the ciprofloxacin formulations, the ciprofloxacin/lipid ratio in the SM/chol vesicles was 5.2- to 5.8-fold greater than that measured for the DPPC/chol vesicles and 2.8- to 3.6-fold greater than that in DSPC/chol LUV.

The altered pharmacokinetics of ciprofloxacin that occurred as a consequence of encapsulation in liposomes (Fig. 3) also significantly altered the biodistribution of ciprofloxacin in liver, lung, spleen, kidney, and muscle after i.v. administration (Fig. 4). The quantities of ciprofloxacin that accumulated in these tissues in mice treated with free antibiotic were maximal at 5 min and then decreased with half-lives in the range of 15 to 30 min (not shown). In contrast, quantities of ciprofloxacin in tissue were substantially higher after administration of all liposomal formulations of the drug (not shown), and the times required for levels of ciprofloxacin in tissue to decrease to 50% of their highest values were in the range of 2 to 4 h in the liver and lung and 3 to 12 h in the spleen and kidney. Consequently, the amounts of ciprofloxacin that accumulated in these tissues during the 24 h after administration were increased by 1.5- to 73-fold (Fig. 4A). An example of the effect of encapsulation on the quantities of ciprofloxacin in the lung is shown in Fig. 4B. The increased drug retention properties of SM/chol vesicles (Fig. 3) were reflected in higher quantities of ciprofloxacin in tissue following i.v. administration of this formulation compared to those with the DPPC/chol and DSPC/chol formulations (Fig. 4A).

Pharmacokinetics after i.p. and intratracheal administrations. The substantial improvements in the pharmacokinetics and biodistribution of liposomal ciprofloxacin, compared to free ciprofloxacin, after i.v. administration suggested that similar advantages might be conferred by other routes of administration. Consequently, the pharmacokinetics of free and liposomal (SM/chol) formulations of ciprofloxacin after i.p. and intratracheal administration were also examined.

Quantities of ciprofloxacin in plasma decreased rapidly after the administration of free ciprofloxacin by either the i.v. or i.p. route (Fig. 5A). As described above (Fig. 3A), the half-life of free ciprofloxacin after both i.v. administration and i.p. administration was approximately 0.25 h. In contrast, the administration of liposomal ciprofloxacin by both the i.v. and i.p. routes resulted in significantly higher concentrations of ciprofloxacin in plasma than after administration of the free antibiotic. As in Fig. 3, the half-life of the SM/chol formulation of liposomal ciprofloxacin after i.v. administration was increased to 2.7 h, representing a greater than 10-fold increase in the half-life as a consequence of encapsulation. After i.p. administration of liposomal ciprofloxacin, the concentrations of drug in plasma gradually increased during the first 4 h and then exhibited pharmacokinetics identical to those of the i.v.-administered formulations between 4 and 24 h (Fig. 5A). The amounts of

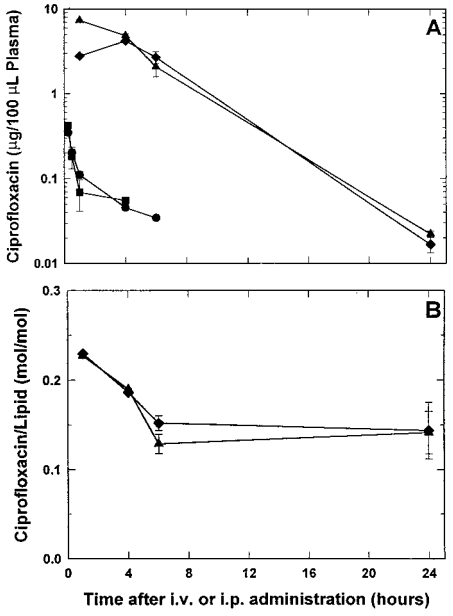

FIG. 5. Comparison of ciprofloxacin pharmacokinetics after i.v. and i.p. administration. (A) Levels of free (●,■) or liposomal (▲,◆) ciprofloxacin in plasma after i.v. (●,▲) or i.p. (■,◆) administration. Liposomes were composed of SM/chol, and the dose of ciprofloxacin injected for all groups was 15 mg/kg. (B) Ciprofloxacin/lipid ratios in plasma after i.v. (▲) or i.p. (◆) administration of ciprofloxacin encapsulated in SM/chol liposomes. Data represent means ± standard errors from three mice.

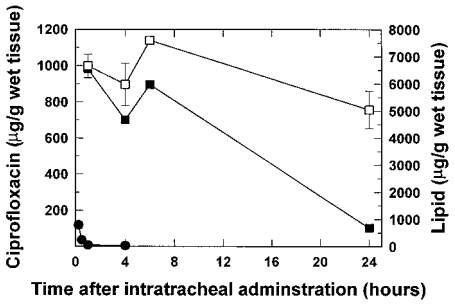

FIG. 6. Pharmacokinetics after intratracheal administration. Quantities of ciprofloxacin (●,■) and lipid (□) in the lungs of ICR mice after the intratracheal administration of either free ciprofloxacin (●) or ciprofloxacin encapsulated in liposomes comprised of DPPC/chol (■,□) are shown. Data represent means ± standard errors from three mice.

ciprofloxacin retained within SM/chol LUV were identical after either i.v. or i.p. administration (Fig. 5B).

Free ciprofloxacin was rapidly cleared from the lungs after intratracheal administration, with a half-life of 0.21 h (Fig. 6), whereas encapsulation dramatically increased the retention of drug at this site. Specifically, the half-lives for ciprofloxacin in the lungs were increased to 6.9 h (DPPC/chol), 14.6 h (DSPC/chol), and 23.6 h (SM/chol), representing increases of 33-, 69-, and 112-fold, respectively (Fig. 6 and data not shown). Ciprofloxacin longevity in lung tissue was a direct consequence of the retention of the vesicles at this site and the retention of the drug in the vesicles (Fig. 6 and data not shown). At 24 h, the quantities of lipid remaining in the lungs ranged from 76 to 106% of the initial dose, indicating that the carriers did not extravasate to the circulation, but remained in the lung tissue and acted as slow-release reservoirs of ciprofloxacin. The superior retention of ciprofloxacin by SM/chol LUV resulted in a significant increase in drug quantities in the lungs over 24 h compared to those of free drug and the more leaky DPPC/chol and DSPC/chol formulations. The levels of lipid in the plasma after intratracheal administration of liposomal ciprofloxacin were negligible (data not shown), confirming that the liposomes were not able to extravasate from the lung tissue to the circulation. As expected, levels of ciprofloxacin in the plasma after intratracheal administration of liposomal ciprofloxacin were also negligible, consistent with the short half-life of free drug that diffuses into the circulation from the vesicles trapped in the airways.

In vivo antibacterial efficacy of free and liposomal ciprofloxacin. The antibacterial activities of free and liposomal ciprofloxacin have been compared in a *Salmonella typhimurium* infection model. The SM/chol formulation of liposomal ciprofloxacin was chosen for the efficacy studies because of its superior drug retention properties in vivo (Fig. 3). In these experiments, mice were infected with 66 to 88 CFU of *Salmonella typhimurium*, and then 24 h later, they were given an i.v. bolus of free or liposomal (SM/chol) ciprofloxacin or free ciprofloxacin plus empty SM/chol LUV. The ciprofloxacin doses administered were either 1 or 20 mg/kg. Results are summarized in Fig. 7. Free ciprofloxacin, administered at either dose, had only minor effects on the numbers of viable bacteria recovered from the livers and spleens of infected animals in comparison to those recovered from controls (Fig. 7). Liposomal ciprofloxacin at a drug dose of 1 mg/kg reduced the number of viable bacteria in the liver and spleen by approximately 10- to 100-fold compared to the number reduced by free ciprofloxacin at the same dose (data not shown). At 20 mg/kg, the numbers of viable bacteria remaining in the livers and spleens of infected animals were 10^3- to 10^4-fold lower in those treated with encapsulated ciprofloxacin than those in control animals (untreated or saline treated) or animals treated with the same dose of free ciprofloxacin in the presence or absence of empty SM/chol carriers (Fig. 7 and data not shown). Finally, it should be added that single i.v. administration of the SM/chol formulation of liposomal ciprofloxacin resulted in an approximate doubling of the survival time for mice bearing this *Salmonella typhimurium* infection.

An additional control experiment was performed to ensure that the reduction in the number of viable bacteria in the organs of the infected animals that had been treated with liposomal ciprofloxacin was not due to the release of ciprofloxacin from liposomes during tissue homogenization. Homogenates of livers obtained from control animals or from animals treated with liposomal ciprofloxacin 5 days earlier were centrifuged to remove particulate material. The supernatants (containing any remaining liposomes or ciprofloxacin released from the liposomes during homogenization) were filter sterilized with a 0.22-μm-pore diameter filter, and then $3 \cdot 10^6$ CFU of *Salmonella typhimurium* SL1344 in PBS were added to 1 ml of PBS or to 1 ml of each of the liver suspension filtrates. These bacterial suspension-organ filtrates were incubated for 1 h at 4°C, and then 100 μl of 10^0- to 10^5-fold dilutions were assayed for viable bacteria. Neither free nor liposomal ciprofloxacin treatments reduced the number of bacteria in spleen or liver homogenates (not shown). Therefore, the bactericidal efficacy of the liposomal ciprofloxacin occurred postadministration in the mice and did not occur as an artifact of organ-associated ciprofloxacin that was plated onto the microbiological growth media.

DISCUSSION

Ciprofloxacin loading. The most prominent examples of active drug loading into liposomes for the purpose of drug delivery are doxorubicin (44) and vincristine (3). Both are lipophilic amines and are loaded into vesicles which possess an acidic interior with respect to the external solution. Accumulation proceeds because lipophilic weak bases (a characteristic of a surprising number of drugs) distribute across the liposomal membrane according to the relationship $[\text{drug}]_{IN}/[\text{drug}]_{OUT} = [H^+]_{IN}/[H^+]_{OUT}$ (5). Consequently, a transmembrane ΔpH of 3 U (inside acidic) will result in drug loading until, at equilib-

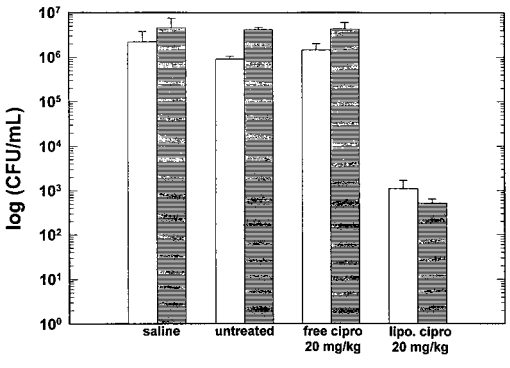

FIG. 7. In vivo antibacterial efficacy against *Salmonella typhimurium*. The CFU of viable bacteria per milliliter of liver (open bars) or spleen (shaded bars) homogenates for animals infected with 66 to 88 CFU of *Salmonella typhimurium* were either left untreated, were treated once with saline, or were treated once with free or liposomal ciprofloxacin at 20 mg/kg. Data represent means ± standard errors from three mice.

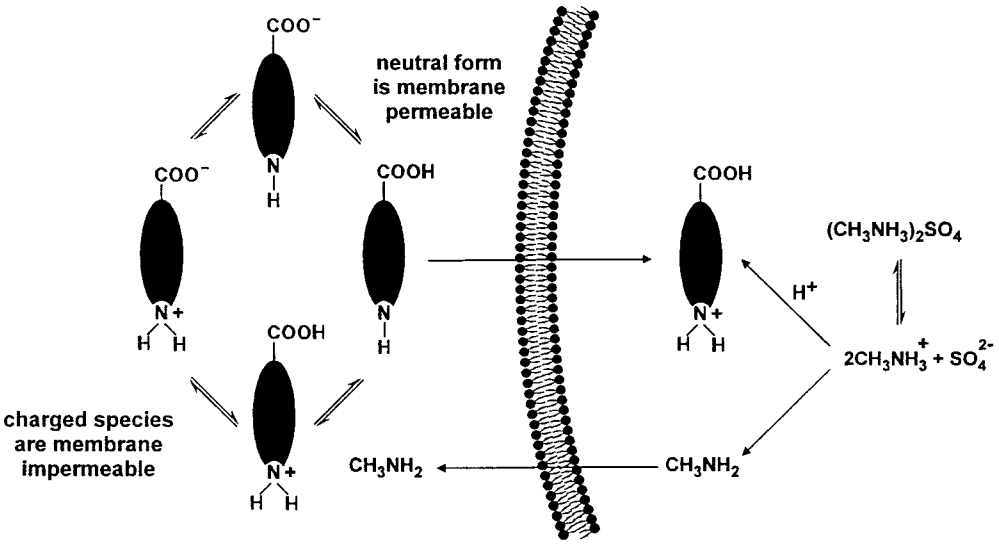

FIG. 8. Schematic representation of the active loading method for ciprofloxacin. Ciprofloxacin exists in cationic, anionic, zwitterionic, and neutral forms. (Note that this ionization scheme is identical both inside and outside the liposome, but for clarity, is shown only on the outside.) Inside the liposome is a low internal pH as a consequence of methylammonium ionization to methylamine and a proton. The neutral form of ciprofloxacin is membrane permeable and crosses to the aqueous lumen of the liposomes, where the low pH favors the protonated species. Cationic ciprofloxacin cannot diffuse back out of the vesicle; consequently, the drug accumulates until $[drug]_{IN}/[drug]_{OUT} = [H^+]_{IN}/[H^+]_{OUT}$. As a proton is consumed by each neutral drug species, equilibrium is maintained by disassociation of methylammonium into methylamine and a proton. This accounts for the observed 1:1 molar stoichiometry between drug uptake and methylamine efflux.

rium, there is a 1,000-fold-higher concentration of drug inside the vesicle than outside.

The practical advantages of the process have enabled the successful clinical development of several liposome-based drug delivery systems. Active loading is simple and efficient. Conditions can be selected such that 100% of the drug can be effectively encapsulated into preformed LUV, therefore minimizing process development issues. Moreover, the presence of a transmembrane ion gradient helps retain drugs inside carriers during storage and after administration. Most drugs for which active loading has been characterized are lipophilic cations or anions (20). Therefore, given the zwitterionic nature of ciprofloxacin, it was not obvious that this drug could be loaded by an active process. However, recently we (15) and others (19, 34) demonstrated that ciprofloxacin accumulation occurred in response to an ammonium sulfate ion gradient, and in this report, we have demonstrated that rapid and complete uptake is also achieved with a methylamine sulfate chemical gradient (Fig. 2). A scheme is presented in Fig. 8 which describes the uptake process and explains the characteristics of ciprofloxacin loading we observed. Encapsulated methylamine sulfate ionizes into methylammonium and sulfate. The methylammonium ion further disassociates into methylamine and a proton, both of which can escape the vesicle by diffusing through the bilayer down their concentration gradients. The bilayer is relatively impermeable to sulfate ions. Furthermore, the efflux of protons is limited by the rapid formation of a transmembrane potential (negative inside) caused by movement of the positive charge out of the vesicles. As methylamine continues to diffuse from the vesicle, the internal concentration of protons increases until the membrane potential and ΔpH are in equilibrium (Fig. 8).

Ciprofloxacin possesses both a carboxyl function and an amino function. At a pH of <6, the molecule exhibits a net positive charge, whereas for pHs of >9, the charge is net negative (15, 45). Above or below these pH extremes, ciprofloxacin is very soluble, but over the physiological pH range, the drug is practically insoluble (Fig. 1). The four ionization states for ciprofloxacin are shown in Fig. 8. In general, charged molecules cannot cross the bilayer at a significant rate; consequently, it is reasonable to assume that it is the uncharged species that diffuses into the vesicle down its concentration gradient. Inside, the low internal pH favors the protonated ciprofloxacin species, trapping the drug which is unable to diffuse back across the bilayer in the charged form. Drug accumulates inside the vesicle (stoichiometrically with the efflux of methylamine [Fig. 2B]) until an electrochemical equilibrium is reached such that $[drug]_{IN}/[drug]_{OUT} = [H^+]_{IN}/[H^+]_{OUT}$ (5, 13). However, it should be noted that it is an oversimplification to assume that the transmembrane drug distribution exactly reflects the ΔpH; factors such as the membrane-water partitioning coefficient for the drug and precipitation with internal counterions also need to be taken into consideration (5). As long as the internal concentration of methylamine sulfate exceeds that of the drug, then at equilibrium, a stable, internal acidic pH will be maintained, as was observed in this study.

Ciprofloxacin has also been actively loaded into unilamellar liposomes in response to a transmembrane gradient of ammonium sulfate in a procedure dependent on the pH of the external solution (19, 34). These workers concluded that the protonated species of ciprofloxacin is the membrane-permeable form of the drug (34) and, once diffused across the membrane, is rendered impermeable by precipitation within the liposome interior (19, 40). However, analysis of liposome-encapsulated ciprofloxacin by proton NMR does not support the conclusion that intraliposomal ciprofloxacin exists as a ciprofloxacin sulfate precipitate (49a). Rather, the upfield shift of the aromatic proton resonances associated with the encapsulated drug is more consistent with ciprofloxacin self-association.

Pharmacokinetics, biodistribution, and efficacy. The longevity of ciprofloxacin in plasma was considerably increased by encapsulation in 100-nm LUV (Fig. 3A). SM/chol LUV had the greatest drug retention in vivo compared to DSPC/chol or DPPC/chol LUV, and this was reflected in the drug/lipid ratio measured in the blood over 24 h (Fig. 3B). The observation

that SM/chol vesicles were better able to retain actively loaded drug than glycerol-based phospholipid-cholesterol-containing vesicles has been made previously with formulations of vincristine (48). The data from the latter study suggested that SM/chol vesicles are more stable in blood and consequently can maintain the ΔpH required to keep the drug entrapped.

Levels of ciprofloxacin in tissue following i.v. administration of encapsulated formulations reflected the biodistribution of the liposomal carrier systems. It is well known that phospholipid vesicles accumulate in organs of the RES. We analyzed liver, spleen, lung, and kidney tissues, and in all cases, encapsulated ciprofloxacin was readily measured out to 4 or 6 h postinjection. In comparison, free drug was cleared quickly, and was undetectable after 1 h according to our assay protocol (Fig. 4B). Interestingly, i.p. administration of encapsulated ciprofloxacin gave rise to a profile for the drug in blood almost identical to that obtained after i.v. delivery (Fig. 5A); the only difference in clearance kinetics was seen during the first 4 h as vesicles drained into the circulation via the lymphatics. The drug/lipid ratio data (Fig. 5B) indicated that during this time, the release of encapsulated ciprofloxacin proceeded at the same rate as that for vesicles administered i.v. In contrast, vesicles introduced into the lungs via tracheal intubation were well retained at the epithelium-air interface. As a result, a reservoir of ciprofloxacin can be maintained in the lung for at least 24 h (Fig. 6A), with free drug being steadily released into surrounding tissue. It is worth making the point that because the formulations described here are stable in solution, they are readily aerosolized and therefore could be targeted directly to lung tissue. This would represent a novel route of administration for ciprofloxacin. Aerosolized formulations of ciprofloxacin have not been developed, in part because of the drug's poor solubility and extremely bitter taste. However, the actively loaded formulations described here overcome both of these limitations. Because ciprofloxacin is 100% encapsulated and is held inside the delivery system by a ΔpH, the drug can be suspended in physiological media without precipitation at concentrations that far exceed the free drug solubility. Furthermore, encapsulation would be expected to mask the bitter taste. The potential for aerosolized delivery of ciprofloxacin may have clinical significance in the light of recent data demonstrating that formulations of liposomal ciprofloxacin were highly effective, following intratracheal administration, in treating mice whose lungs had been inoculated with *Francisella tularensis*, a virulent respiratory pathogen (49).

The systemic *Salmonella typhimurium* infection used here is one in which the infecting bacteria seed primarily in the liver and spleen, and, furthermore, at least 88% of the liver-resident bacteria are localized within macrophages (39). We have demonstrated significantly enhanced efficacy of encapsulated ciprofloxacin against these intracellularly localized bacteria in vivo. The drug was administered as a single dose i.v. and compared to an equivalent dose of free ciprofloxacin. Given the superior drug retention properties of the SM/chol vesicles, we chose to test this formulation in the infection model. At 20 mg/kg, the free antibiotic exhibited very little activity, but an equivalent dose of encapsulated ciprofloxacin reduced the viable bacterial count in both the liver and spleen by 3 to 4 orders of magnitude (Fig. 7). Presumably the increased activity over that of the free drug was due to a combination of increased drug concentrations in the circulation and the accumulation of a ciprofloxacin reservoir in the target organs. Taken in sum, these data highlight the versatility and efficacy of the liposomal ciprofloxacin formulations.

ACKNOWLEDGMENTS

We thank Sharon Ruschkowski, Agneta Richter-Dahlfors, and Brett Finlay for cooperation with the in vivo efficacy experiments and Norbert Maurer for performing the ^{13}C-NMR analyses.

REFERENCES

1. **Bermudez, L. E., A. O. Yao-Young, J.-P. Lin, J. Cogger, and L. S. Young.** 1990. Treatment of disseminated *Mycobacterium avium* complex infection of beige mice with liposome-encapsulated aminoglycosides. J. Infect. Dis. **161:** 1262–1268.
2. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
3. **Boman, N. L., M. B. Bally, P. R. Cullis, L. D. Mayer, and M. S. Webb.** 1995. Encapsulation of vincristine in liposomes reduces its toxicity and improves its anti-tumor efficacy. J. Liposome Res. **5:**523–541.
4. **Boman, N. L., D. Masin, L. D. Mayer, P. R. Cullis, and M. B. Bally.** 1994. Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing P388 tumors. Cancer Res. **54:**2830–2833.
5. **Cullis, P. R., M. J. Hope, M. B. Bally, T. D. Madden, L. D. Mayer, and D. B. Fenske.** 1997. Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles. Biochim. Biophys. Acta **1331:**187–211.
6. **Di Ninno, V. L., J. W. Cherwonogrodzky, and J. P. Wong.** 1993. Liposome-encapsulated ciprofloxacin is effective in the protection and treatment of BALB/c mice against *Francisella tularensis*. J. Infect. Dis. **168:**793–794.
7. **Düzgüneş, N., D. R. Ashtekar, D. L. Flasher, N. Ghori, R. J. Debs, D. S. Friend, and P. R. J. Gangadharam.** 1991. Treatment of *Mycobacterium avium-intracellulare* complex infection in beige mice with free and liposome-encapsulated streptomycin: role of liposome type and duration of treatment. J. Infect. Dis. **164:**143–151.
8. **Düzgüneş, N., V. K. Perumal, L. Kesavalu, J. A. Goldstein, R. J. Debs, and P. R. J. Gangadharam.** 1988. Enhanced effect of liposome-encapsulated amikacin on *Mycobacterium avium-M. intracellulare* complex infection in beige mice. Antimicrob. Agents Chemother. **32:**1404–1411.
9. **Fenlon, C. H., and M. H. Cynamon.** 1986. Comparative in vitro activities of ciprofloxacin and other 4-quinolones against *Mycobacterium tuberculosis* and *Mycobacterium intracellulare*. Antimicrob. Agents Chemother. **29:**386–388.
10. **Fresta, M., A. Spadaro, G. Cerniglia, I. M. Ropero, G. Puglisi, and P. M. Furneri.** 1995. Intracellular accumulation of ofloxacin-loaded liposomes in human synovial fibroblasts. Antimicrob. Agents Chemother. **39:**1372–1375.
11. **Gay, J. D., D. R. DeYoung, and G. D. Roberts.** 1984. In vitro activities of norfloxacin and ciprofloxacin against *Mycobacterium tuberculosis*, *M. avium* complex, *M. chelonei*, *M. fortuitum*, and *M. kansasii*. Antimicrob. Agents Chemother. **26:**94–96.
12. **Harrigan, P. R., M. J. Hope, T. E. Redelmeier, and P. R. Cullis.** 1992. Determination of transmembrane pH gradients and membrane potentials in liposomes. Biophys. J. **63:**1336–1345.
13. **Harrigan, P. R., K. F. Wong, T. E. Redelmeier, J. J. Wheeler, and P. R. Cullis.** 1993. Accumulation of doxorubicin and other lipophilic amines into large unilamellar vesicles in response to transmembrane pH gradients. Biochim. Biophys. Acta **1149:**329–338.
14. **Hope, M. J., M. B. Bally, G. Webb, and P. R. Cullis.** 1985. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution trapped volume and ability to maintain a membrane potential. Biochim. Biophys. Acta **812:**55–56.
15. **Hope, M. J., and K. F. Wong.** 1995. Liposomal formulation of ciprofloxacin, p. 121–134. *In* P. N. Shek (ed.), Liposomes in biomedical applications. Harwood Academic Publishers, Germany.
16. **Hwang, K. J.** 1987. Liposome pharmacokinetics, p. 109–156. *In* M. J. Ostro (ed.), Liposomes: from biophysics to therapeutics. Marcel Dekker, Inc., New York, N.Y.
17. **Klemens, S. P., M. H. Cynamon, C. E. Swenson, and R. S. Ginsberg.** 1990. Liposome-encapsulated-gentamicin therapy of *Mycobacterium avium* complex infection in beige mice. Antimicrob. Agents Chemother. **34:**967–970.
18. **Kresta, A., P. N. Shek, J. Odumeru, and J. M. A. Bohnen.** 1993. Distribution of free and liposome-encapsulated cefoxitin in experimental intra-abdominal sepsis in rats. J. Pharm. Pharmacol. **45:**779–783.
19. **Lasic, D. D., B. Ceh, M. C. A. Stuart, L. Guo, P. M. Frederik, and Y. Barenholz.** 1995. Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery. Biochim. Biophys. Acta **1239:**145–156.
20. **Madden, T. D., P. R. Harrigan, L. C. L. Tai, M. B. Bally, L. D. Mayer, T. E. Redelmeier, H. C. Loughrey, C. P. S. Tilcock, L. W. Reinish, and P. R. Cullis.** 1990. The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. Chem. Phys. Lipids **53:**37–46.
21. **Magallanes, M., J. Dijkstra, and J. Fierer.** 1993. Liposome-incorporated ciprofloxacin in treatment of murine salmonellosis. Antimicrob. Agents Chemother. **37:**2293–2297.
22. **Majumdar, S., D. Flasher, D. S. Friend, P. Nassos, D. Yajko, W. K. Hadley, and N. Düzgüneş.** 1992. Efficacies of liposome-encapsulated streptomycin and ciprofloxacin against *Mycobacterium avium-M. intracellulare* complex

infections in human peripheral blood monocyte/macrophages. Antimicrob. Agents Chemother. **36:**2808–2815.
23. **Mayer, L. D., M. B. Bally, P. R. Cullis, S. L. Wilson, and J. T. Emerman.** 1990. Comparison of free and liposome encapsulated doxorubicin tumor drug uptake and antitumor efficacy in the SC115 murine mammary tumor. Cancer Lett. **53:**183–190.
24. **Mayer, L. D., M. B. Bally, H. Loughrey, D. Masin, and P. R. Cullis.** 1990. Liposomal vincristine preparations which exhibit decreased drug toxicity and increased activity against murine L1210 and P388 tumors. Cancer Res. **50:** 575–579.
25. **Mayer, L. D., T. D. Madden, M. B. Bally, and P. R. Cullis.** 1993. pH gradient-mediated drug entrapment in liposomes, p. 27–44. *In* G. Gregoriadis (ed.), Liposome technology, vol. II. Entrapment of drugs and other materials. CRC Press, Boca Raton, Fla.
26. **Mayer, L. D., D. Masin, R. Nayar, N. L. Boman, and M. B. Bally.** 1995. Pharmacology of liposomal vincristine in mice bearing L1210 ascitic and B16/BL6 solid tumors. Br. J. Cancer **71:**482–488.
27. **Mayer, L. D., R. Nayar, R. L. Thies, N. L. Boman, P. R. Cullis, and M. B. Bally.** 1993. Identification of vesicle properties that enhance the antitumour activity of liposomal vincristine against murine L1210 leukemia. Cancer Chemother. Pharmacol. **33:**17–24.
28. **Mayer, L. D., and G. St.-Onge.** 1995. Determination of free and liposome-associated doxorubicin and vincristine levels in plasma under equilibrium conditions employing ultrafiltration techniques. Anal. Biochem. **232:**149–157.
29. **Mayer, L. D., L. C. L. Tai, M. B. Bally, G. N. Mitilenes, R. S. Ginsberg, and P. R. Cullis.** 1990. Characterization of liposomal systems containing doxorubicin entrapped in response to pH gradients. Biochim. Biophys. Acta **1025:**143–151.
30. **Mayer, L. D., L. C. L. Tai, D. S. C. Ko, D. Masin, R. S. Ginsberg, P. R. Cullis, and M. B. Bally.** 1989. Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. Cancer Res. **49:**5922–5930.
31. **Nacucchio, M. C., M. J. Gatto Bellora, D. O. Sordelli, and M. D'Aquino.** 1988. Enhanced liposome-mediated antibacterial activity of piperacillin and gentamicin against gram-negative bacilli *in vitro*. J. Microencapsulation **5:** 303–309.
32. **Nayar, R., M. J. Hope, and P. R. Cullis.** 1989. Generation of large unilamellar vesicles from long-chain saturated phosphatidylcholines by extrusion technique. Biochim. Biophys. Acta **986:**200–206.
33. **Nightingale, S. D., S. L. Saletan, C. E. Swenson, A. J. Lawrence, D. A. Watson, F. G. Pilkiewicz, E. G. Silverman, and S. X. Cal.** 1993. Liposome-encapsulated gentamicin treatment of *Mycobacterium avium-Mycobacterium intracellulare* complex bacteremia in AIDS patients. Antimicrob. Agents Chemother. **37:**1869–1872.
34. **Oh, Y.-K., D. E. Nix, and R. M. Straubinger.** 1995. Formulation and efficacy of liposome-encapsulated antibiotics for therapy of intracellular *Mycobacterium avium* infection. Antimicrob. Agents Chemother. **39:**2104–2111.
35. **Omri, A., M. Ravaoarinoro, and M. Poisson.** 1995. Incorporation, release and *in vitro* antibacterial activity of liposomal aminoglycosides against *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **36:**631–639.
36. **Onyeji, C. O., C. H. Nightingale, and M. N. Marangos.** 1994. Enhanced killing of methicillin-resistant *Staphylococcus aureus* in human macrophages by liposome-entrapped vancomycin and teicoplanin. Infection **22:**338–342.
37. **Paganoni, R., C. Herzog, A. Braunsteiner, and P. Hohl.** 1988. Fleroxacin: in-vitro activity worldwide against 20,807 clinical isolates and comparison to ciprofloxacin and norfloxacin. J. Antimicrob. Chemother. **22:**3–17.
38. **Pick, U.** 1981. Liposomes with a large trapping capacity prepared by freezing and thawing of sonicated phospholipid mixtures. Arch. Biochem. Biophys. **212:**186–194.
39. **Richter-Dahlfors, A., A. M. J. Buchan, and B. B. Finlay.** 1997. Murine salmonellosis studies by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. J. Exp. Med. **186:**569–580.
40. **Rodriguez, J. L., S. Z. Trooskin, R. S. Greco, R. A. Herbstman, A. P. Donetz, and R. A. Harvey.** 1986. Reduced bacterial adherence to surfactant-coated catheters. Curr. Surg. **43:**423–425.
41. **Rottenberg, H., T. Grunwald, and M. Avron.** 1972. Determination of (delta)pH in chloroplasts. 1. Distribution of [^{14}C]methylamine. Eur. J. Biochem. **25:**54–63.
42. **Sanders, C. C., W. E. Sanders, Jr., and R. V. Goering.** 1987. Overview of preclinical studies with ciprofloxacin. Am. J. Med. **82:**2–11.
43. **Sherphof, G. L., F. Kuipers, J. T. P. Denkser, H. H. Spanyer, and R. J. Wonk.** 1987. Liposomes *in vivo*: conversion of liposomal cholesterol to bile salts. Biochem. Soc. Trans. **15:**625–631.
44. **Tardi, P. G., N. L. Boman, and P. R. Cullis.** 1996. Liposomal doxorubicin. J. Drug Targeting **4:**129–140.
45. **Teixeira, M. H. S. F., L. F. Vilas-Boas, V. M. S. Gil, and F. Teixeira.** 1995. Complexes of ciprofloxacin with metal ions contained in antacid drugs. J. Chemother. **7:**126–132.
46. **Vaage, J., D. Donovan, E. Mayhew, P. Uster, and M. Woodle.** 1993. Therapy of mouse mammary carcinomas with vincristine and doxorubicin encapsulated in sterically stabilized liposomes. Int. J. Cancer **54:**959–964.
47. **Vladimirsky, M. A., and G. A. Ladigina.** 1982. Antibacterial activity of liposome-entrapped streptomycin in mice infected with *Mycobacterium tuberculosis*. Biomedicine **36:**375–377.
48. **Webb, M. S., T. O. Harasym, D. Masin, M. B. Bally, and L. D. Mayer.** 1995. Sphingomyelin-cholesterol liposomes significantly enhance the pharmacokinetic and therapeutic properties of vincristine in murine and human tumour models. Br. J. Cancer **72:**896–904.
49. **Wong, J. P., J. W. Cherwonogrodzky, V. L. Di Ninno, R. De la Cruz, and E. G. Saravolac.** 1995. Liposome-encapsulated ciprofloxacin for the prevention and treatment of infectious diseases caused by intracellular pathogens, p. 105–120. *In* P. N. Shek (ed.), Liposomes in biomedical applications. Harwood Academic Publishers, Germany.
49a. **Wong, K. F., et al.** Submitted for publication.
50. **Zou, Y. Y., Y. H. Ling, S. Reddy, W. Priebe, and R. Perez-Soler.** 1995. Effect of vesicle size and lipid composition on the *in vivo* tumor selectivity and toxicity of the non-cross-resistant anthracycline Anamycin incorporated in liposomes. Int. J. Cancer **61:**666–671.