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Intracellular Accumulation of Ofloxacin-Loaded Liposomes in Human Synovial Fibroblasts

MASSIMO FRESTA,[1] ANGELO SPADARO,[1] GIUSEPPA CERNIGLIA,[2] INMACULADA MORAGA ROPERO,[2] GIOVANNI PUGLISI,[1] AND PIO M. FURNERI[2]*

Institute of Pharmaceutical and Toxicological Chemistry,[1] and Institute of Microbiology,[2] University of Catania, Catania, Italy

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In order to incorporate ofloxacin within liposomes, the reverse-phase evaporation technique was carried out. The liposome lipid matrix consisted of dipalmitoylphosphatidylcholine-cholesterol-dihexadecylphosphate (4:3:4 molar ratio). The liposome formulation presented a mean size of 185 ± 31 nm and had an encapsulation capacity of 5.3 μl/μmol. The liposome formulation was able to deliver ofloxacin into McCoy cells in a greater amount (2.6-fold) than the free drug, improving antibiotic accumulation.

The efficacies of fluoroquinolone antibiotics have led to their proposed use for the treatment and prophylaxis of different bacterial diseases: therapy for the respiratory tract, skin structure, bone and gastrointestinal infections, as well as urinary tract infections. Owing to their poor solubility in water, fluoroquinolones are used as salts (lactates or hydrochlorides). This problem may be overcome by formulating solid dispersion systems that are able to improve the dissolution time and the bioavailability of the drug (7, 11). Therefore, the use of antibiotic delivery systems would result in enhanced concentrations of the antimicrobial agent at the site of infection, for the following reasons: (i) targeting of drug to the infected tissues and (ii) increased intracellular antibiotic concentrations and (iii) reduced toxicities of potentially toxic antibiotics resulting from the targeting of antimicrobial drugs to the infectious organism. Liposomes are possible carriers for controlled drug delivery and targeting by the intravenous route.

Liposomes are microscopic vesicles consisting of multiple concentric lipid bilayers formed when lipid films are dispersed in an aqueous medium. As with most drug carriers, liposomes have been used extensively in an attempt to improve the selective delivery and the therapeutic index of antimicrobial agents (3, 31) and anticancer drugs (19, 32). Liposomes, artificial phospholipid membranes, are usually produced (as in the case of the present study) from naturally occurring, biodegradable, and nontoxic lipids such as lecithin, cholesterol, and phosphatidylserine. The use of antibiotic-loaded liposomes may result in increased antibiotic concentrations at the site of infection (passive or active targeting), increased intracellular antibiotic concentrations (enhanced liposome-cell interaction), and reduced toxicities of potentially toxic antimicrobial agents (biodistribution, away from host cell) (1). After in vivo administration via the intravenous route, conventional liposomes are taken up by the reticuloendothelial system, and hence, they may be used as antibiotic carriers for the treatment of infections involving the RES (8, 9). To deliver biologically active agents outside the RES, liposome biodistribution may be changed by manipulation of vesicle size and phospholipid composition, in this way achieving a prolonged circulation time in the blood (20).

The aim of the study described here was to investigate the uptake of ofloxacin-loaded liposomes into human synovial fibroblasts (McCoy cells) compared with that of the free drug. Preliminary experiments demonstrated that McCoy cells showed poor antibiotic accumulation (18). On the basis of those results, we studied the interaction of these cells with ofloxacin-loaded liposomes in order to investigate the enhancement of the intracellular drug owing to the liposomal bilayer-microbial membrane interaction. Ofloxacin is a fluoroquinolone antibiotic that has excellent in vitro antibacterial activity against many microorganisms (34).

Cholesterol was purchased from Sigma Chemical Co. (St. Louis, Mo.). 1,2-Dipalmitoyl-*sn*-glycero-phosphocholine monohydrate and dihexadecyl hydrogen phosphate were obtained from Fluka Chemical Co. (Buchs, Switzerland). The lipid purity (greater than 99%) was assayed by two-dimensional thin layer chromatography (14, 17). Ofloxacin was kindly provided by Sigma-Tau s.p.a. (Pomezia, Italy). The purity of this drug was greater than 99.5%, as assayed by high-pressure liquid chromatography (HPLC) (28).

In order to prepare the liposomes, the reverse-phase evaporation technique was carried out (12): the lipids, dissolved in chloroform-methanol (3:1; vol/vol), and ofloxacin, dissolved in methanol (final concentration, 1.4 mM), were dried in a round-bottom flask under nitrogen with a rotary evaporator. The resulting thin lipid film was dissolved in ethyl ether. Isotonic (pH 7.4) phosphate-buffered saline (PBS) was added to this solution, and the resulting mixture was sonicated at 10°C for 20 min (Bransonic model 2200). The ether remaining in the emulsion was removed by evaporation under reduced pressure at room temperature, achieving a turbid, white reverse-phase evaporation vesicle (REV) suspension with a lipid concentration of 50 $mg \cdot ml^{-1}$. Morphological and dimensional characterizations were carried out by freeze-fracture electron microscopy and a light-scattering technique, respectively (16).

The unloaded drug was separated from the liposome colloidal suspension by gel permeation chromatography (Fig. 1). An aliquot (1 ml) of the REV suspension was loaded onto a Sepharose 4B fine column (1.5-cm diameter, 50-cm length; Pharmacia, Uppsala, Sweden). Chromatographic separation was carried out at room temperature at a flow rate of 160 μl/min, and fractions of 2.4 ml were collected. Detection was

* Corresponding author. Mailing address: Istituto di Microbiologia, Università di Catania, Via Androne 81, 95124 Catania, Italy. Phone: 39 95 316038 or 39 95 316201. Fax: 39 95 316201.

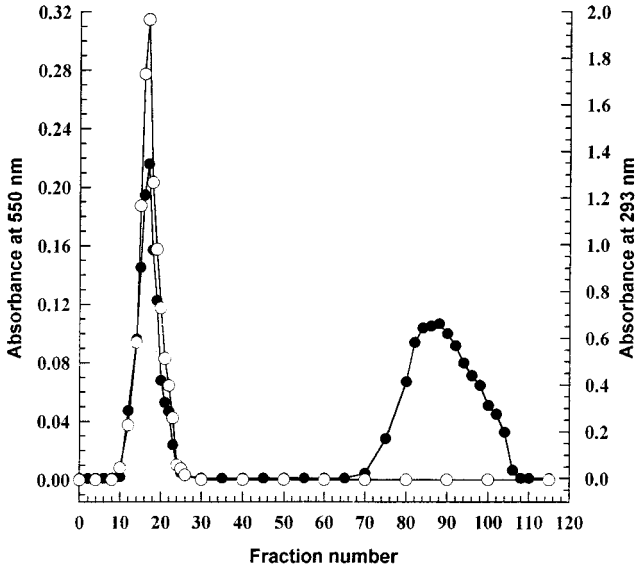

FIG. 1. Gel permeation chromatograph of ofloxacin-loaded REV colloidal suspensions (1 ml) at a lipid concentration of 50 mg · ml^{-1}. The mobile phase (PBS) was delivered at a flow rate of 160 μl/min. The liposome suspension was eluted in the void volume, whereas the entrapped drug was retained by the gel. The drug quantity was determined by UV-visible analysis at 293 nm (●) after liposome destruction with Triton X-100. The turbidity measurement was carried out at 550 nm (○).

carried out by a turbidity assay at 550 nm. In addition, UV-visible measurements of the various samples were performed at 293 nm (ofloxacin λ_{max}). The cloudy fractions containing liposomes were treated with Triton X-100 (5%; wt/vol) to clear them. The encapsulation values are expressed as encapsulation capacity (EC) and percent trapping efficiency (TE) (5, 15). The EC was calculated from the ratio between the concentration of the drug entrapped (in micromoles per microliter) and that of the added drug (in micromoles per microliter) times the lipid concentration (in micromoles per microliter) of the liposome suspension. TE is the percentage of the amount of starting drug which became entrapped.

Human synovial fibroblast cells (McCoy cells) were maintained in Dulbecco modified Eagle medium (DMEM; Biochrom KG-Seromed, Berlin, Germany) with 10% fetal calf serum (Biochrom KG-Seromed). Cells were split once weekly. The cells were dislodged by using trypsin, placed in six-well plates at approximately 10^6 cells per well, and incubated for 24 h before use. Confluent cells (10^6 cells per well) were washed with DMEM, and 5.7 μg of each drug formulation per ml was added. After various incubation periods, the cells were washed in warm PBS and were lysed with chilled distilled water (2 ml for each well), and the cells were then frozen, thawed, and sonicated for 5 min.

The aqueous samples containing the lysed cells were sonicated for 5 min and filtered (0.2-μm-pore-size Teflon membrane; Spartan-3 Schleicher & Schuell, Keene, N.H.), and then an appropriate amount of a solution of internal standard (timolol; Sigma) was added; they were then analyzed by a new HPLC method. The apparatus consisted of a Varian Star 9010 solvent delivery pump (Varian Associates Inc., Walnut Creek, Calif.) with a 100-μl loop, a Varian Star 9050 variable UV-visible detector, and a Varian 4400 reporting integrator. Chromatographic separation was carried out on a LiChrospher 100 RP-18 reverse-phase C_{18} analytical column (5 μm, 250 by 4 mm [inner diameter]; Hewlett-Packard, Milan, Italy) with a

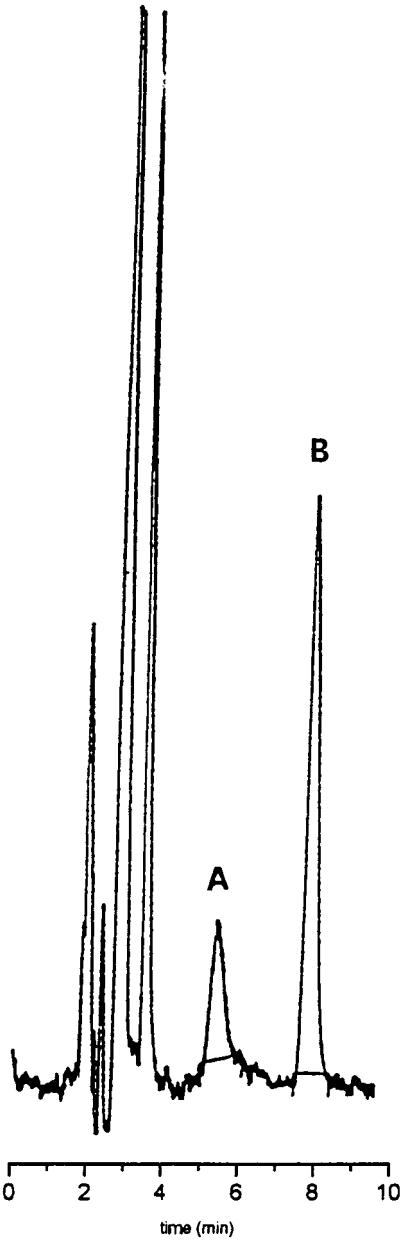

FIG. 2. Typical high-pressure liquid chromatogram of a cell sample. (A) Ofloxacin (33.2 ng/ml); retention time, 5.40 min. (B) Internal standard (timolol); retention time, 7.70 min.

LiChrospher 100 RP-18 (Hewlett-Packard) direct-connect guard column (5 μm; 4 by 4 mm). The mobile phase, which was 0.5% triethylamine (Merck, Darmstadt, Germany) in water (pH 2.5) with H_3PO_4 (78% w/w; Merck)–CH_3CN (82:18 [vol/vol]; LiChrosolv; Merck), was delivered at a flow rate of 1.0 ml/min. Detection was carried out at 293 nm, and internal standardization was used. Figure 2 shows a representative chromatogram of ofloxacin and the internal standard. The reproducibility of the assay was determined by repetitive analysis of McCoy cell suspension spiked with 20, 120, and 350 ng of ofloxacin standard per ml. The within-day reproducibilities were 95.3% ± 6.8%, 96.7% ± 4.3%, and 98.2% ± 2.1% (assay accuracy ± relative standard deviation; $n = 6$), respectively. The day-to-day reproducibilities were 98.1% ± 3.2%, 102.3%

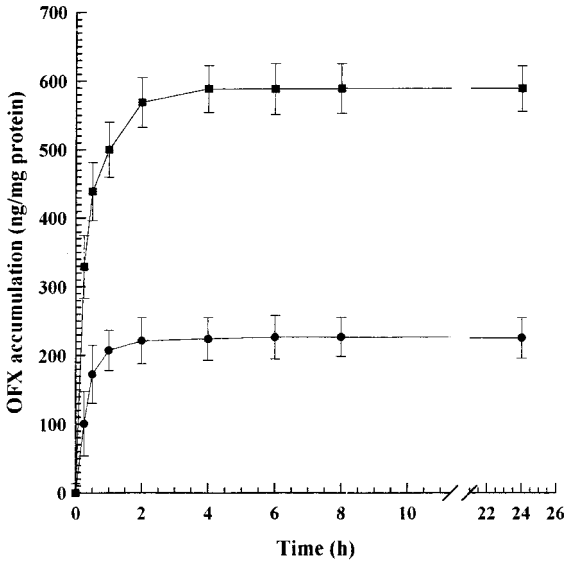

FIG. 3. Time course of ofloxacin accumulation in McCoy cells. The biological assay was carried out at room temperature (20°C) by adding 5.7 μg of free (●) or liposome-entrapped (■) ofloxacin (OFX) per ml to confluent McCoy cells. The amount of drug was determined by HPLC. Each point represents the average ± standard deviation of nine different experiments.

± 1.8%, and 98.3% ± 2.2%, respectively. The lower limit of detection for the assay in McCoy cells was approximately 18 ng/ml, for a signal/noise ratio of 3:1. No interfering peaks were observed in the blank cell chromatogram.

Preliminary results (13) showed that ofloxacin-loaded liposomes were at least twofold more active than the free drug against *Escherichia coli* ATCC 25922 and ATCC 35218, *Enterococcus faecalis* ATCC 29213, and *Staphylococcus aureus* ATCC 29212. In particular, the lipid composition dipalmitoylphosphatidylcholine-cholesterol-dihexadecylphosphate (4:3:4 molar ratio) resulted in the best antimicrobial effectiveness. The presence of cholesterol was necessary to ensure liposome stability either in vitro or in vivo. In fact, high-density lipoproteins rapidly destroy the liposome structure, allowing the entrapped drug to leak out (12). The REV preparation procedure mainly led (over 85%) to the formation of large unilamellar vesicles with high trapping efficiencies. The mean REV size was 185 ± 31 nm, with a polydispersity index of 0.09. REV presented EC and TE values of 5.3 μl/μmol and 16.2%, respectively.

The accumulation profiles of free and liposome-entrapped ofloxacin are shown in Fig. 3. Both curves are typically biphasic, presenting a first step of very rapid drug accumulation and then a flat phase. The key parameters describing the accumulation kinetics showed that for both ofloxacin dosage forms the accumulation reached the maximum concentration of drug in cells at approximately 120 min, after which there was a plateau phase. The maximum concentration of drug in cells was significantly ($P < 0.01$) greater for ofloxacin in loaded liposome suspensions than for the free drug (588 versus 221 ng/mg of protein, respectively, a 2.6-fold increase).

Intracellular penetration of antimicrobial agents is presumably the main factor that may be capable of determining biological effectiveness in the treatment of infectious diseases caused by microorganisms which can survive after phagocytosis (e.g., *Legionella pneumophila*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *S. aureus*) (23, 35). To be effective against these microorganisms, antibiotics should be able not only to enter into cells but also to maintain their antibacterial activities within the cellular environment. Fluoroquinolones, as well as clindamycin, erythromycin, and other antimicrobial agents, are capable of penetrating polymorphonuclear leukocytes (10, 23, 24, 27, 30) and exerting their biological action. Discrepancies in experimental results among studies with different drugs are caused by differences in antimicrobial activity and binding (21). Measurement of the intracellular antibiotic concentration is based on the determination of the total amount of drug associated with the cell. The drug may be more or less tightly distributed in the various cell components (i.e., membrane, cytoplasmic region, nuclear components) without being present at the site where the intracellular microorganism resides. If the antimicrobial drug is tightly linked to intracellular components, a poor intracellular activity may result, although the drug concentration within cells is abundantly greater than the minimal effective concentrations in blood. In fact, poor intracellular accumulation and unfavorable intracellular conditions, such as acidic pH, may shift the MICs toward higher concentrations. Fluoroquinolones are less active at acidic pH values, and thus, their intracellular activities are hampered because of the low pH in lysosomes. For these reasons, liposomes represent a valid tool for delivering antimicrobial agents (fluoroquinolones) into the cells, ensuring an entrance pathway in addition to the classic ones (2, 4, 22), i.e., the porin pathway and phagocytosis. The liposome formulation may enhance the passage of drug through the biological membrane, avoiding drug metabolization and inactivation up to the time of drug entrance and action. In fact, liposomes may ensure drug passage by means of cell surface adsorption and diffusion, biological membrane fluidization and consequent permeabilization, and bilayer-membrane fusion (25, 26, 29, 33). The liposome formulation was able to deliver higher amounts of ofloxacin into cells than the amounts delivered by a simple drug solution, as shown in Fig. 3, and may be capable of ensuring different drug entrance pathways. These properties offer another possible advantage of the liposome formulation, namely, the possibility of rendering microorganisms which normally show fluoroquinolone resistance susceptible by making modifications to the outer bacterial membrane. Furthermore, the possibility of formulating liposomes that circulate for long periods of time (12, 20) and that may passively target the drug should be taken into account. In fact, it is difficult for liposomes to achieve extravasation through capillary fenestration except when alteration of the capillary integrity occurs. In this way the compromised capillary structure leads to enhancement of blood vessel permeability, such as in the case of acute meningitis, in which the increased capillary permeability is associated with an increase in the concentrations of various antibiotics in the cerebrospinal fluid (6). Tissue infection is normally accompanied by inflammatory reactions within the same tissue. These reactions alter the physicochemical environment, thereby enhancing the regional capillary permeability and allowing the passage of the liposomes armed with the antimicrobial agents (ofloxacin). Therefore, either a passive target process or protection of the drug from the circulating enzymes leading to its biotransformation and elimination is achieved; thus, the drug remains active for a much longer period.

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