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Small particle aerosols of enviroxime-containing liposomes

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Summary

Enviroxime inhibits the replication of all rhinoviruses tested in vitro at very low concentrations (10–100 ng/ml), but evaluations in humans have not consistently shown efficacy. Lack of an appropriate method for administering this water-insoluble drug may have contributed to the latter result. The present report describes the characteristics and utilization of small particle aerosols to continuously deliver enviroxime-containing liposomes (LE) throughout the respiratory tract. The enviroxime content of liposomes and biological fluids of exposed individuals was quantified by high performance liquid chromatography using C18 resin, a mobile phase of 60:40 acetonitrile:water, and monitoring at 215 nm. Small particle aerosols of LE generated by Puritan-Bennett nebulizers had mass median diameters ranging from 2.4 to 3.1 μm. The concentration of enviroxime in aerosol particles was proportional to the reservoir concentration; during the first hour of operation, the mean concentration was 20 μg of enviroxime/l of aerosol. Liposome particles in the reservoir, although initially heterogeneous in size (<0.1 to >1 μm), were processed by passage through the nebulizer to smaller, more homogeneous particles; the majority were less than 0.2 μm. In a preliminary study to evaluate short term tolerance and toxicity, five volunteers were exposed to small particle aerosol of LE for 1 h. At 1 h post-treatment, large amounts of enviroxime were still present in the nasal wash as determined both by HPLC and biological assay. Enviroxime was not detected in any urine sample and was detected in only 1 of 5 serum samples. No side effects were noted. This data suggest that liposome aerosols offer

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a method for the delivery of hydrophobic compounds for the treatment of respiratory diseases.

Enviroxime; Small particle aerosol; Liposome; Rhinovirus

Introduction

Enviroxime is a potent anti-rhinovirus drug which has been shown to inhibit in vitro the replication of all rhinovirus strains tested (> 80) at concentrations in the range of < 10 to 120 ng/ml (Ahmad and Tyrrell, 1986; Delong, 1984; Delong and Reed, 1980; Ninomiya et al., 1985; Delong et al., 1978; Wu et al., 1978). Clinical trials of both rhinovirus challenge studies and naturally acquired infections have not shown consistent effectiveness of enviroxime when applied topically as a nasal spray with or without an additional oral dose (Hayden and Gwaltney, 1982; Levandowski et al., 1982; Miller et al., 1985; Phillpotts et al., 1981; Phillpotts et al., 1983; Betts et al., 1981). The standard dose for intranasal spray in these studies was 568 μg, four times daily for a total of 2.3 mg. A possible reason for the inconsistency of clinical results was suggested by Phillpotts et al. (1981) who noted that the small oral doses of enviroxime given in some studies had no effect on respiratory infections and that it seemed probable that the spray dosage was low. In addition, the distribution of particles within the respiratory tract was not determined. There was the additional problem of the low solubility of enviroxime in aqueous solution that required the drug to be administered in an alcohol carrier, causing nasal irritation (Hayden and Gwaltney, 1982; Levandowski et al., 1982; Miller et al., 1985; Phillpotts et al., 1981; Phillpotts et al., 1983). To circumvent these problems we developed a method that increased the solubility of enviroxime by incorporating the drug into liposomes while retaining its antiviral activity (Wyde et al., 1988).

Successful delivery of antiviral drugs directly to the site of virus replication in the respiratory epithelium has been demonstrated in several studies involving respiratory syncytial virus infections in infants (Hall et al., 1983; Knight et al., 1986; Taber et al., 1983) and influenza A and B virus infections in adults (Gilbert and Knight, 1986; Knight et al., 1981, 1986; McClung et al., 1983). The advantages of this method of delivery have been discussed (Knight et al., 1986). The present report describes the characteristics and utilization of small particle aerosols to continuously distribute enviroxime-containing liposomes throughout the respiratory tract.

Materials and Methods

Materials

Enviroxime ((E)-6[(hydroxyimino)phenylmethyl]-1-[(1-methylethyl)-sulfonyl]-

1*H*-benzimidazol-2-amine) and ^{14}C-enviroxime (13.8 mCi/mmol [89.7% pure]) were obtained from Eli Lilly and Co. (Indianapolis, IN). Puritan-Bennett nebulizer models #1917 and #1920 were purchased from Puritan-Bennett (Los Angeles, CA). Commercially made enviroxime-containing liposomes composed of egg yolk phosphatidylcholine were obtained from Avanti Polar Lipids, Inc. (Pelham, AL) via our specifications. Chemicals for HPLC analysis were HPLC-grade or as pure as possible. [2-Palmitoyl-9,10^{-3}H(*N*)]-phosphatidylcholine (58 Ci/mmol) was purchased from New England Research Products (Boston, MA).

Quantification of enviroxime

Enviroxime was quantified by high performance liquid chromatography (HPLC) with monitoring at 215 nm (Waters, Milford, MA). All measurements were made at ambient temperature on a Microsorb C18 stainless steel HPLC column (particle size, 5 μm; length, 25 cm; inner diameter, 4.6 mm; Rainin Instrument Co., Emeryville, CA). Mobile phase was acetonitrile:water (60:40) flowing at a rate of 1 ml/min. Under these conditions, enviroxime eluted around 4 min. The standard run was for 10 min with a 10 min mobile phase wash between samples.

Enviroxime content of liposomes and biological fluids

Because of the phosphatidylcholine component of liposomes, it was necessary to separate the enviroxime from the phospholipid before HPLC analysis to prevent binding of the lipid to the C18 column. Separation was accomplished with the use of Waters Sep-Pak C18 cartridge for rapid sample preparation (Waters, Milford, MA). Sep-Pak cartridges were activated sequentially with 10 ml each of absolute methanol, 100% acetonitrile, and water. Separation and recovery of enviroxime from liposome preparations were determined by adding trace amounts of ^{14}C-enviroxime (ca. 3.5×10^5 cpm; 17.8 pmol/cpm) and ^{3}H-phosphatidylcholine (ca. 3.7×10^5 cpm; 42.5 pmol/cpm) prior to making the liposomes. Enviroxime was extracted from the liposome with the use of the Sep-Pak according to the following procedure (Fig. 1): (1) 1–2 ml of liposomes in aqueous solution were added to an activated Sep-Pak and the eluant was discarded (fraction 1); (2) Sep-Pak was washed with 4 ml of water and the eluant was discarded (fraction 2); (3) Sep-Pak was washed with 0.5 ml acetonitrile:water (80:20) and the eluant was discarded (fraction 3), and (4) enviroxime was eluted from the Sep-Pak with 2.0 ml of acetonitrile:water (80:20) (fraction 4 and first half of fraction 5). An aliquot of each liquid fraction, as well as the total resin (fraction 6) and cartridge (fraction 7) itself were mixed with Aquasol scintillation fluid and counted. Enviroxime administered in a liposome was quantified in nasal wash fluids, serum, and urine using these Sep-Pak and HPLC methodologies. In the human volunteer studies, a total of 5–10 ml of water was used for each nasal wash. Biological activity of enviroxime was assayed as previously described (Wyde et al., 1988). The level of sensitivity in in vitro assays was 40 ng/ml.

Phosphatidylcholine content of liposomes

Phosphatidylcholine was calculated from a phosphate analysis (Gerlach and

Deuticke, 1963), using a correction factor of 7.9 mg of egg yolk phosphatidylcholine/mg of phosphate.

Particle characteristics of enviroxime-containing liposome aerosols

Aerosol particle size was determined using a Mark II Andersen 1 sampler (Andersen Samplers Inc., Atlanta, GA). Calculations of aerodynamic mass median diameter and geometric standard deviation were based on gravimetric and chemical determinations of samples collected over a 5-min sampling period. Enviroxime and/or phosphatidylcholine concentrations in an aerosol were determined by collecting a 2-min aerosol sample in all-glass impingers containing 10 ml of water. The all-glass impingers were calibrated to collect 12.5 l of air/min. The efficiency of generating an aerosol of enviroxime or phosphatidylcholine (i.e., spray factor) from the drug preparation was calculated from the concentration of compound in the aerosol (μg/l of air) divided by the concentration in the reservoir (mg/ml of liquid) from which the aerosol was generated.

Electron microscopy of liposomes

Aerosolized liposomes collected in a water trap and reservoir samples diluted to 50 μg of enviroxime/ml were mixed with an equal volume of 2% phosphotungstic acid in phosphate buffer, pH 6.3. A drop was placed on a copper mesh grid coated with Formvar and allowed to air dry. Random sections were selected and photographed for determination of the size of the liposomes before and after aerosolization. Electron microscopy using a JOEL 100 C and size measurements were performed by Dr. Joiner Cartwright, Department of Pathology, Baylor College of Medicine. He was unaware of the experimental protocol.

Quantification of liposome size

Randomly photographed liposome preparations (8, before; 7 after aerosolization) were used to determine change in particle sizes after aerosolization for 1 h. Only those liposomes $\geq$ 100 nm which comprised the majority of the liposome mass were measured. Prior to measuring liposomes, the following ranges of liposome size were arbitrarily selected: 100–250, 250–500, 500–750, >750 nm. The largest diameter was used when liposomes were not uniform.

Results

Quantification of enviroxime in liposomes

Using the methodology for quantifying enviroxime as described in Materials and Methods, enviroxime was detected at 5 ng/10 μl injection volume and had a linear response to at least 500 ng/10 μl injection volume (Fig. 2).

Enviroxime contained within liposomes was separated from the lipid components with the use of Sep-Pak rapid sample preparation cartridges as described in Materials and Methods. Separation and recovery of enviroxime was monitored by adding trace amounts of ^{14}C-enviroxime and ^{3}H-phosphatidylcholine prior to mak-

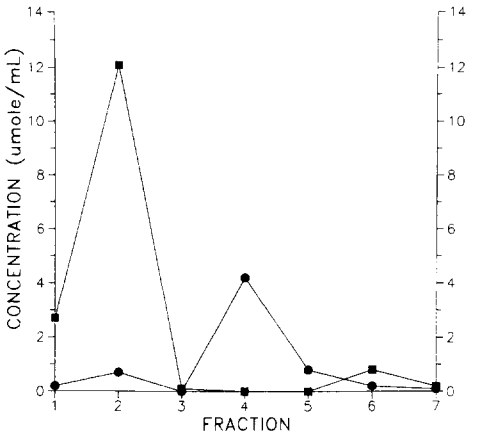

Fig. 1. Extraction of enviroxime from liposomes using Sep-Pak C18 rapid preparation cartridges. Liposomes were prepared with ^{14}C-enviroxime (17.8 pmol/cpm) and [^{3}H]-phosphatidylcholine (42.5 pmol/cpm). Cartridges were activated and elution was performed with ACN:H_2O (80:20) as described in Materials and Methods. Volume of fractions 1–5 (ml): 1–2, 4, 0.5, 1.5, 1.5, respectively. Fraction 6 was total amount associated with the resin and fraction 7 was the total amount associated with the cartridge. Symbols: ●, Enviroxime; ■, Phosphatidylcholine.

ing the liposomes. Using this procedure, 93.6 ± 0.6% of the phosphatidylcholine was recovered in the water wash fraction (Fig. 1, fraction 2), 6.8 ± 0.5% remained associated with the C18 resin (fraction 6) and with the Sep-Pak (fraction 7), and no detectable lipid in the enviroxime fraction (fractions 4 plus 5). Recovery of enviroxime was 81.8 ± 1.7% and it was free of contaminating phospholipid.

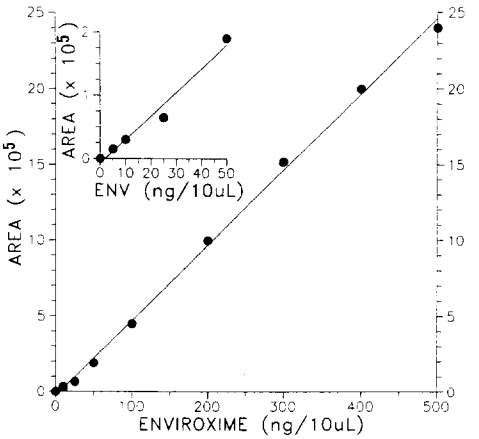

Fig. 2. Enviroxime standard curve. Enviroxime was quantitated by HPLC analysis over a concentration range of 5 to 500 ng injected as described in Materials and Methods. Inset: Enviroxime over the range of 5 to 50 ng injected.

When enviroxime was fractionated using a Sep-Pak and subsequently detected by UV during HPLC analysis, a change in absorption was noted when compared to unprocessed enviroxime used as standard. Thus, recovery of various concentrations of enviroxime from liposomes was undertaken using the above extraction procedure and UV detection. Correcting for the recovery associated with Sep-Pak extraction (81.8%), the recovery of enviroxime incorporated into liposomes (range: 0.4–100 μg/ml) as determined by HPLC analysis was 66.3 ± 9.7% or a total recovery of enviroxime by this procedure of 54.1%.

Characteristics of an enviroxime-containing liposome small particle aerosol

A Collison and two Puritan-Bennett nebulizers were evaluated for the optimization of the output of enviroxime-containing liposomes (LE). The Collison nebulizer had very poor efficiency in producing an aerosol of enviroxime under the conditions used (26 psi, flow rate of 13–15 l/min). Next, we examined two models of the Puritan-Bennett nebulizer. Since our standard volume of liposome preparation for aerosolization was 30 ml, we tested a model with a 60 ml and 250 ml reservoir. The 250 ml reservoir model was tested in two configurations. In the first, the reserve air port was left open as might occur when treating a patient; in the second, the port was closed during aerosolization. Model #1920 (250 ml reservoir) generated heterogeneous aerosol particles of greater aerodynamic mass median diameter (AMMD) than Model #1917 and thus a larger volume. This increase in volume was directly responsible for the greater output of enviroxime in the aerosol during 1 h of aerosolization (mean, 46 vs 26 μg/l, $P = 0.008$; Student's t-test, two-tailed) (Table 1). The aerosol particles behaved as non-hygroscopic particles in that the AMMD did not decrease in the presence of excess air (data not shown). Model 1920 was used in subsequent studies.

The Avanti preparation of LE (5 mg/ml enviroxime and 15 mg/ml phosphati-

TABLE 1

Characteristics of enviroxime-liposome aerosol generated by a Puritan-Bennett nebulizer

Nebulizer	AMMD[1] (μm)	GSD[1] (μm)	Output[2] at 1 h (μg/l)	Spray factor[3]
Model #1917 (60 ml reservoir)	2.4 ± 0.2	2.8 ± 0.2	26 ± 12	1.9 ± 0.6
Model #1920 (250 ml reservoir)				
'Open' port	3.1 ± 0.5	3.1 ± 0.4	49 ± 16	2.7 ± 1.0
'Closed' port			46 ± 16	3.5 ± 1.9

[1] AMMD, aerodynamic mass median diameter; GSD, geometric standard deviation.

[2] P value for difference in output for model #1917 vs model #1920 is 0.014.

[3] Spray factor: Efficiency of aerosol generation is calculated as (μg drug/l of air)/(mg drug/ml in reservoir).

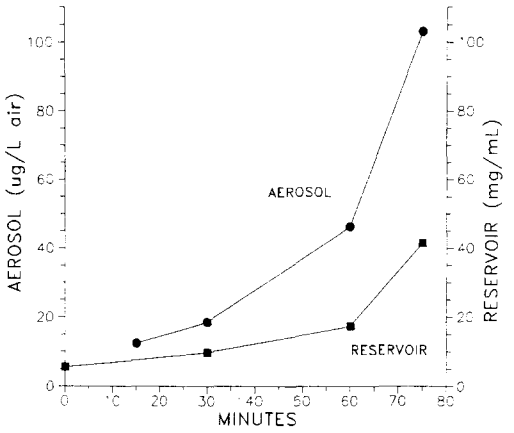

Fig. 3. Kinetics of aerosol generation of enviroxime-containing liposomes. Aerosols were generated with the Puritan-Bennett nebulizer, model #1920, and enviroxime was quantitated by HPLC analysis as described in Materials and Methods. Symbols represent the amount of enviroxime in the aerosol (●) and reservoir (■).

dylcholine in 30 ml of water) was similarly tested to determine the concentrations of enviroxime and phosphatidylcholine in the reservoir and in the aerosol over a period of 90 min or until the liquid was consumed. Over the first 60 min there was a gradual increase in enviroxime concentration in the reservoir and a parallel increase in the output of enviroxime in the aerosol (Fig. 3). After 1 h the remaining liquid in the reservoir (ca. 10–15 ml) evaporated rapidly, leading to a marked increase in the enviroxime concentration in both the reservoir and the aerosol. The mean output of enviroxime for a 1 h period of treatment would be approximately 20 μg/l of aerosol. The concentration of phosphatidylcholine in the aerosol during the 1 h period increased in a parallel manner and was 3–4 times greater than enviroxime (data not shown).

Processing of liposomes during aerosolization

Using a Coulter counter, we observed a shift in the size distribution of the multilamellar liposomes from a more heterogeneous towards a smaller, more homogeneous population of particles. To quantify this shift in size, particles were observed with an electron microscope. The most numerous particles both before and after aerosolization were smaller than 100 nm in diameter with many being about 25–75 nm in diameter. However, liposomes ≥ 100 nm comprised the majority of the liposome mass. Before aerosolization the liposome preparation consisted of particles of heterogeneous size, ranging from 100 to 750 nm with only 37% of the particles in the size range of 100–250 nm (Table 2). However, following 1 h of aerosolization, 91% of the liposome particles were now in that size range, reflecting a shift to a more homogeneous population as observed by the Coulter counter measurements.

TABLE 2

Change in distribution of liposome size following aerosolization

	Particle size range (nm)			
	100–250	250–500	500–750	>750
Before aerosolization	37[1]	40	22	1
After aerosolization	91	8	2	0

[1] Percent of total particles ≥ 100 nm in diameter. Eight and seven photographs randomly taken before and after aerosolization, respectively, were measured. A total of 218 and 53 liposome particles before and after aerosolization, respectively, were measured.

Quantification of enviroxime in biological fluids following aerosol exposure of volunteers

In a preliminary study to evaluate short-term tolerance and toxicity, five adult male volunteers were exposed to the standard LE preparation for 1 h. From 5 to 60 min following exposure enviroxime could be qualitatively detected by HPLC in nasal wash specimens and retained biological activity (Table 3). Enviroxime was detected in only one of five samples and was not found in any urine specimens. The only comment noted by the volunteers was the presence of minor nasal discharge during aerosolization which ceased immediately following exposure.

TABLE 3

Quantification of enviroxime in biological fluids following a one hour aerosol exposure of five volunteers with 5 mg/ml enviroxime-liposome preparation[a]

Volunteer	Nasal wash			Serum		Urine	
	Time[b] (min)	HPLC[b] (μg)	Biological[c] (titer)	HPLC[d] (ng/ml)	Biological[e] (titer)	Time[f] (h)	HPLC[g] (ng/ml)
A	5	84	ND[h]	0	0	0.5,1	0
B	5	227	128	0	0	1,6.5	0
C	15	750	>256	0	0	1,5	0
D	30	159	>256	0	0	1	0
E	60	612	>256	159	0	1	0

[a] Enviroxime dosage: 1.2 mg/h/l of aerosol. One can calculate the expected dose that a person would receive in the following manner: average concentration in the aerosol × min volume × duration of treatment × 0.7 retention factor or for an average 10 l minute volume, the average dose of enviroxime retained would be 8.4 mg (1.2 mg/h/l × 10 l × 0.7).

[b] Time post-treatment at which nasal wash was obtained. Recovered volume (ml) of nasal wash: A, 3; B, 1; C, 9; D, 4; E, 7.

[c] Maximum titer of antirhinovirus activity as determined on KB cells with two-fold dilutions.

[d] Minimum level of detection of free enviroxime at end of 1 h of treatment: 50 ng/ml of serum.

[e] -Fold rise in pre- vs. post-treatment serum antirhinovirus activity as determined on KB cells with two-fold dilutions.

[f] Time(s) from start of treatment that urine samples were collected.

[g] Minimum level of detection of free enviroxime: 50 ng/ml of urine.

[h] ND, not done.

Discussion

It was our evaluation that the low solubility of enviroxime in aqueous solutions (1–2 μg/ml) accounted for some of the variability in efficacy observed in clinical trials of rhinovirus infections. The utilization of liposomes composed of only enviroxime and egg yolk phosphatidylcholine has allowed us to increase the concentration of enviroxime to 5 mg/ml of liposome preparation. The incorporation of enviroxime into the lipid bilayer appears to stabilize the liposome in a similar manner as does the incorporation of cholesterol (unpublished data). Quantification of enviroxime in these liposome preparations was achieved by separating the enviroxime from the phospholipid and measuring the content by HPLC analysis employing UV monitoring. While this method was simple and rapid for determining enviroxime concentrations in liposomes and in nasal wash samples, it lacks the sensitivity to measure the low levels that may occur in some biological fluids. Since enviroxime is rapidly cleared by the liver, conjugated and excreted in the bile, concentrations should be very low in serum and urine. The HPLC system described in this manuscript is capable of detecting enviroxime in serum and urine at a concentration of 50 ng/ml. Use of an electrochemical detector has been shown to be useful in detecting enviroxime in serum and urine at concentrations of 4 ng/ml and 20 ng/ml, respectively (Bopp and Miner, 1982).

Enviroxime-containing liposomes (LE) could be used to generate small particle aerosols similar to those generated previously for the antiviral drug, ribavirin (Knight et al., 1986). With liposomes, however, the size of the aerosol particle is larger (2.4–3.1 μm) than that measured with ribavirin (1.3–1.6 μm) (Knight et al., 1986) and acts as a non-hygroscopic particle which does not change in size in the presence of excess dry air. Since these particles do not enlarge in increased relative humidity, they will deposit in the nose to about the same extent as aqueous ribavirin particles which enlarge to an AMMD of 3.4 μm on inhalation. Since rhinovirus replication is more restricted to the nasopharynx, LE should be deposited upon the cells of the respiratory epithelium where infection is centered.

During the mechanics of generating the aerosol, it was noted that the size of the liposome particles decreased to become more homogeneous. Similar results have been reported by Farr et al. (1985). We also observed that the efficiency of generating an aerosol of enviroxime or phosphatidylcholine was not the same. The efficiency of phospholipid aerosol generation (i.e., spray factor of 9) was similar to that measured for ribavirin using a Collison nebulizer system to generate small particle aerosols (Knight et al., 1986). However, enviroxime efficiency was about one third that of the phosphatidylcholine (i.e., spray factor of 1.9–3.5). It is possible that the shearing forces generated within the nebulizer during aerosolization disrupt the phospholipid bilayer creating smaller liposome particles which contain less enviroxime. Further characterization of the mechanics of liposome aerosol generation is needed.

Because a suitable animal model was not available to study rhinovirus infections and to gain some preliminary data prior to actual clinical trials, five adult male volunteers were exposed to aerosols of LE for a period of one hour to evaluate short

term tolerance and toxicity. Monitoring the volunteers indicated no adverse effects from this exposure. A one hour treatment protocol was selected to give a deposited dose in the respiratory tract of 7–10 mg of enviroxime for an average minute volume of 8–12 l [(20 μg/l, average concentration in aerosol) (0.13–0.20 l/h, hour volume) (1 h, duration of treatment) (0.7, retention factor) (Knight et al., 1986)]. This amount of enviroxime, estimated to be in the range of 100 μg/ml of respiratory secretion, is in considerable excess over that shown to be necessary to inhibit rhinovirus in vitro (10–100 ng/ml) (Ahmad and Tyrrell, 1986; DeLong, 1984; DeLong and Reed, 1980; Ninomiya et al., 1985). The actual amount of enviroxime will depend on the uptake of drug by the respiratory epithelium and on the rate of clearance. Previously we have shown that liposomes tagged with a fluorescence marker accumulated with time within the tall columnar epithelium lining the bronchi and bronchioles (Wyde et al., 1988). Opposing the presence of enviroxime is mucociliary clearance which appears to remove these drug-containing liposomes from the nose and lungs at a fairly rapid rate (Wyde et al., 1988). Additional studies in man will be necessary to determine the efficacy of LE in the treatment of natural rhinovirus infections and the role of mucociliary clearance on respiratory drug levels. These studies will require regulatory approval for further aerosolization of enviroxime-containing liposomes in man.

While the current methodology was developed for increasing the concentration of enviroxime for delivery to the respiratory tract, it is applicable to many water-insoluble compounds which might have potential antiviral activity if given in sufficient dosage. In addition to increasing the solubility of a compound, incorporation of some drugs into liposomes decreases their toxicity without significantly altering their therapeutic effect. This was observed with enviroxime (Wyde et al., 1988), as well as with amphotericin B (Mehta et al., 1984) and streptomycin (Tadakuma et al., 1985). We feel that liposome aerosols offer a method for the delivery of a variety of drugs, especially hydrophobic compounds, for the treatment of respiratory disease.

Acknowledgements

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