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Research Paper

Oral bioavailability enhancement of a hydrophilic drug delivered via folic acid-coupled liposomes in rats

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Abstract

Objectives A liposome preparation that is amenable to receptor-mediated endocytosis has been developed to enhance the oral bioavailability of poorly absorbable peptidomimetic drugs by use of folic acid as the mediator of liposomal uptake.

Methods Folic acid was physically coupled to the surface of the liposomes and cefotaxime was used as the model drug. In-vivo evaluation was carried out on eight Sprague-Dawley rats in a two-way crossover study to compare the oral bioavailability of cefotaxime loaded in folic acid-free liposomes and folic acid-coupled liposomes. Blood samples were collected from the tail vein and plasma cefotaxime levels were determined using an HPLC method.

Key findings Enhanced oral bioavailability ($AUC_{0-\infty}$) of cefotaxime was observed when administered via folic acid-coupled liposomes. The peak plasma concentration (C_{max}) of cefotaxime was increased when administered via folic acid-coupled liposomes as compared with folic acid-free liposomes. At 90% confidence interval, the value for $AUC_{0-\infty}$ was 1.4–2-times higher and the value for C_{max} was 1.2–1.8-times higher for the folic acid-coupled liposomes compared with folic acid-free liposomes.

Conclusions Folic acid could enhance the uptake of liposomally entrapped drug. It could be a useful candidate to supplement liposome delivery systems.

Keywords cefotaxime; folic acid; liposomes

Introduction

Oral liposomal drug delivery has not been fully accepted due to the erratic nature of the results obtained. Many approaches have been investigated to overcome the problem of the variable absorption of the liposome–drug preparations.[1–4] Amongst the techniques investigated is the use of cellular nutrients as mediators of liposomal uptake. This current research has utilised folic acid as the cellular nutrient. Folic acid is an essential nutrient necessary for nucleotide synthesis in which uptake into the cell occurs via receptor-mediated endocytosis.[5,6] Folic acid has extremely favourable biochemical, chemical and physical properties that make it an ideal candidate for this study. These include its high affinity for the cell surface receptors, low immunogenicity, small molecular size, stability in different solvents, low cost and availability.[5,7–9] Folate uptake is believed to occur in all dividing cells at reasonable rates and it is deposited into cytosolic and not lysosomal compartments, making it a suitable ligand to be used for improving the intestinal absorption of peptidomimetic drugs.[10]

Folate was investigated as a possible targeting agent in formulations upon the discovery that folate receptors were over-expressed in many types of cancer cells.[11,12] Most information regarding folate-targeted liposome delivery has been derived from experiments performed *in vitro*.[8,13–16] Moreover, all these studies involved linking folic acid to the liposome surface via chemical conjugation. This would give rise to a new chemical entity which may warrant toxicological study to evaluate its safety profile. Hence, physical coupling of folic acid to liposomes might be a better approach and was used in this study, in which the folic acid was just mixed with the drug-loaded liposomes. Physical coupling is an inexpensive and simple method to couple the folic acid to the liposomes. Thus, the aim

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of this study was to investigate the oral bioavailability enhancement effect of folic acid-coupled liposomes using cefotaxime as the model drug.

Materials and Methods

Materials

Pro-lipo duo (Lucas Meyer, Champlan, France), a specially formulated pro-liposome containing 50% of negatively-charged unsaturated soybean phosphatidylcholine was used. Cefotaxime sodium was obtained from Orchid Chemicals (Alathur, India). Folic acid was purchased from BASF Takeda (Osaka, Japan). Triton X-100 (t-octylphenoxypolyethoxyethanol) was purchased from Sigma (St Louis, Missouri, US). All solvents and chemicals used were of analytical reagent or high-performance liquid chromatography (HPLC) grade.

Preparation of folic acid-free and folic acid-coupled liposomes containing cefotaxime

Pro-lipo duo was used as received. This pro-liposome mixture was converted to concentrated cefotaxime-loaded liposome suspension by dropwise addition of cefotaxime drug solution (20 mg/ml) with continuous stirring at room temperature (22°C) for 30 min. The concentrated liposomes were then gradually diluted with distilled water to yield the folic acid-free preparation containing 5 mg/ml cefotaxime. The proportion of pro-lipo duo : drug solution : distilled water was 1 : 2 : 5, w/w/w, as recommended by the manufacturer.

To obtain the folic acid-coupled preparation, the concentrated cefotaxime-loaded liposome suspension was similarly prepared, but was diluted with a 0.2 mM folic acid aqueous suspension instead of distilled water. The volume of the folic acid suspension used was similar to that of the distilled water used for diluting the concentrated liposome suspension (when preparing the folic acid-free product). Thus, both preparations had similar volume and similar cefotaxime concentration, namely 5 mg/ml. Both preparations were prepared extemporaneously and were used as prepared.

The low molar concentration of folic acid suspension was selected according to the hypothesis of Reddy and Low[17], which suggested that it was sufficient 'to successfully mediate the liposome uptake by folate receptor-bearing cells'.

Particle size analysis

The particle size of the folic acid-free and folic acid-coupled liposomes were estimated by photon correlation spectroscopy (Zetasizer 1000 HS, Malvern, Worcestershire, UK). The distilled water used for dispersing the liposomes was filtered through a 0.22-μm, white GSWP, 47-mm Millipore filter (Bedford, Massachusetts, US). A total of three batches for each formulation were prepared and three measurements were taken on two separate samples from each batch. The Z average diameter, which is a natural intensity weighted mean, and the polydispersity index were used as parameters of mean particle size and size distribution, respectively.

Entrapment efficiency determination

Entrapment efficiency is defined as the percent fraction of the total input drug encapsulated in the liposomes, at a particular phospholipid concentration.[18] It was expressed as mg cefotaxime entrapped per 100 mg phospholipid. The percentage of drug entrapped in the liposomes was calculated using the following formula:

$$\% \text{ entrapped} = ([\text{total cefotaxime}] - [\text{free cefotaxime}])/[\text{total cefotaxime}] \quad (1)$$

Separation of cefotaxime-loaded liposomes from free cefotaxime

The cefotaxime-containing liposomes were separated from the free (unentrapped) cefotaxime by ultracentrifugation at 215 000g and 20°C for 2 h (Beckman Optima L-80, Fullerton, California, US). Duplicate samples were used. The supernatant consisted of free cefotaxime which was collected and kept frozen at −20°C until analysis.

Assay of the free and total cefotaxime

Triton X-100 1% (w/w) was added to the liposome suspension at a 1 : 1 ratio (v/v) to destroy the phospholipid bilayer structures, freeing the drug. The mixture was vortexed for 30 s (Barnstead/Thermolyne, Dubuque, Iowa, US) followed by centrifugation for 15 min at 12 800g (Eppendorf, Hamburg, Germany). Duplicate samples were used. Clear supernatant was transferred to a new microcentrifuge tube and kept frozen at −20°C until analysis. The concentrations of cefotaxime were analysed using an HPLC method reported by Ling *et al*.[19]

In-vivo study protocol

The experimental procedure was approved by the Ethics Committee on Animal Studies, Universiti Sains, Malaysia. The study was conducted according to a two-way crossover design using eight adult male, Sprague-Dawley rats (235–340 g; mean = 279 g, SD = 26 g). The rats were randomly divided into two groups of four rats each. The animals were fasted overnight before each phase of the experimental procedure, while water was supplied freely. They were administered with the preparations according to the sequence shown in Table 1. A one-week wash-out was allowed between the phases of the study.

Both preparations had a volume of 3.2 ml and were administered intragastrically by oral intubation after a 12-h overnight fast. After administration of the preparations an additional 1.0 ml water was given. No food was given throughout the duration of the experiment but the rats were allowed free access to water. The animals were subsequently placed in restraining cages. Blood samples, approximately 0.5 ml, were collected from the tail vein into heparinised microcentrifuge tubes at 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 7 and 9 h post-administration. The blood samples were centrifuged for 20 min at 12 800g. A sample of plasma, approximately 0.2–0.3 ml, obtained from each sample was

Table 1 Sequence of liposome administration

Group	Sequence of administration	
	First week	Second week
1	Folic acid-free liposomes	Folic acid-coupled liposomes
2	Folic acid-coupled liposomes	Folic acid-free liposomes

transferred into a new microcentrifuge tube. All plasma samples were stored frozen at −20°C until analysis.

Analysis of plasma cefotaxime concentration

The concentrations of plasma cefotaxime were determined using the HPLC method reported by Ling *et al.*[19]

Data and pharmacokinetic analysis

The bioavailability of the two preparations were compared using the pharmacokinetic parameters, total area under the plasma concentration–time curve ($AUC_{0-\infty}$), peak plasma concentration (C_{max}) and time to reach peak plasma concentration (T_{max}). Both the C_{max} and T_{max} values were obtained directly from the plasma concentration data, while the $AUC_{0-\infty}$ was calculated by adding the area from time zero to the last sampling time (AUC_{0-t}) and the area from the last sampling time to infinity ($AUC_{t-\infty}$).[20] The former was calculated using the trapezoidal formula and the latter by extrapolating the last measurable plasma concentration to the time axis.

Statistical analysis

For the in-vitro studies, the Student's two sample independent *t*-test was used to determine the difference in mean particle sizes as well as the percentage of cefotaxime entrapped between the two preparations.[21]

As for the in-vivo study, the values of $AUC_{0-\infty}$ and C_{max} obtained from the two preparations were analysed statistically using an analysis of variance procedure appropriate for a crossover design. The $AUC_{0-\infty}$ and C_{max} values were logarithmic transformed before the analysis. The T_{max} values for both of the treatments were compared using the Wilcoxon Signed Rank test for paired samples. A statistically significant difference was considered at $P < 0.05$.

Results

Particle size and entrapment efficiency

Table 2 shows the Z average diameter values and the polydispersity index of the folic acid-coupled and folic acid-free drug-loaded liposomes. The percentage of cefotaxime entrapped by both formulations is also shown in Table 2. The values of the Z average diameter, polydispersity index and percentage of drug entrapped obtained with the two preparations were comparable and not significantly different ($P > 0.05$). Thus, it can be inferred from the results that the addition of folic acid to the liposomes did not cause any significant change in the particle size and size distribution or the entrapment efficiency. However, the rather high polydispersity index values for both liposomal preparations were explained by Ling *et al.*[22] as due to the 'addition of the drug, which may affect the homogeneity of the size of the liposomes produced'.

Table 2 Characteristics of the liposome preparations

Formulation	Particle size		Entrapment efficiency % entrapped
	Z average diameter	Polydispersity index	
Folic acid-coupled liposomes	309.4 ± 2.9	0.36 ± 0.01	22.1 ± 0.6
Folic acid-free liposomes	315.8 ± 5.3	0.38 ± 0.01	27.7 ± 0.6

Values are mean ± SEM.

In-vivo comparative bioavailability study

The mean plasma concentration versus time profiles of cefotaxime administered entrapped in the folic acid-coupled liposomes and folic acid-free liposomes are shown in Figure 1. It was apparent from the plots that the plasma levels of cefotaxime were consistently higher when administered with the folic acid-coupled liposomes as compared with the folic acid-free preparation.

Table 3 shows the mean $AUC_{0-\infty}$, C_{max} and T_{max} values obtained with liposomally-loaded cefotaxime with and without incorporation of folic acid. Table 3 also includes the 90% confidence interval (CI) values for the ratio of $AUC_{0-\infty}$ and C_{max} of cefotaxime administered with folic acid-coupled liposomes over those of the folic acid-free preparation. When the values obtained with the two treatments were analysed using the analysis of variance procedure, it was found that the $AUC_{0-\infty}$ of the folic acid-coupled preparation was significantly higher ($P < 0.01$) than that obtained with the folic acid-free preparation. Likewise, the C_{max} value was significantly higher ($P < 0.05$).

The calculated 90% CI value for $AUC_{0-\infty}$ given in Table 3 showed that the overall oral bioavailability of cefotaxime was increased 1.4–2.0-fold when administered via the folic acid-coupled preparation. For C_{max}, the corresponding

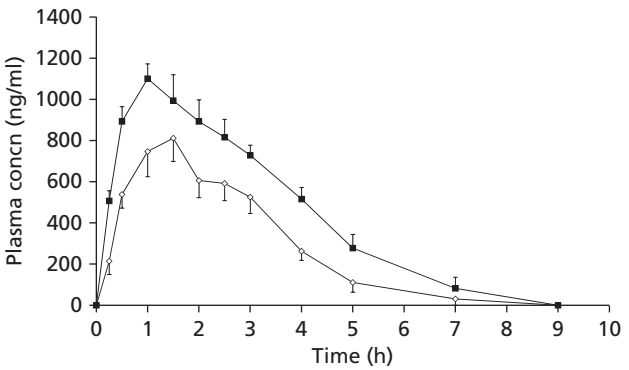

Figure 1 Plasma concentration versus time profiles of liposomally entrapped cefotaxime. Values are mean ± SEM, $n = 8$. ◇ Folic acid-free liposomes; ■ folic acid-coupled liposomes; both were administered orally.

Table 3 Pharmacokinetic parameters of the liposome preparations

Formulation	$AUC_{0-\infty}$ (ng/ml h)	C_{max} (ng/ml)	T_{max} (h)
Cefotaxime-liposomes	2517.1 ± 956.8	830.0 ± 310.6	1.31 ± 0.37
Folic acid-cefotaxime-liposomes	4004.7 ± 937.8	1166.2 ± 306.1	1.06 ± 0.32
CI	1.4–2.0	1.2–1.8	

Values are the mean ± SEM. CI, the 90% confidence interval value of liposomes coupled with folic acid over that without folic acid. $AUC_{0-\infty}$, total area under the plasma concentration–time curve; C_{max}, peak plasma concentration; T_{max}, time to reach peak plasma concentration.

90% CI was between 1.2 to 1.8, indicating that the increase in C_{max} was as pronounced as the $AUC_{0-\infty}$.

There was no statistically significant difference ($P > 0.05$) between the T_{max} values of the two treatments when analysed using the Wilcoxon Signed Rank test. Thus, it appeared that when folic acid was added only the extent of absorption of cefotaxime-loaded liposomes was significantly increased and not the rate.

Discussion

Folic acid is known to enter cells by receptor-mediated endocytosis via a high affinity and high specificity folate receptor.[5] Hence, folic acid has been exploited to deliver macromolecules into the cytoplasm of cells with expressed folate receptors. The affinity of folic acid for the folate receptors remains unaltered when it is covalently attached to a macromolecule via its γ-carboxylate moiety and the conjugate produced can be internalised into the cells by the natural folate endocytosis pathway.[10,13,23] However, direct linkage of folic acid to drug molecules suffers from the possibility of a loss in activity of the drug molecule resulting from the chemical modification, especially for drugs with protein or peptide structures. In addition, the number of drugs delivered at each folate receptor could be amplified by entrapping the drugs within a delivery vehicle such as liposomes. Hence, the use of folic acid as a 'Trojan horse' to deliver folate-tagged liposomes bearing diverse cargo represents a potential drug delivery system for protein and peptidomimetic drugs.[24]

Previous studies using folate-targeted liposomes involved covalent conjugation of folic acid to liposomes via various spacers.[8,13–16] A long intervening spacer seemed to be required to mediate association of folate-conjugated liposomes with receptor-bearing cells, as observed by Lee and Low[13] using KB cells (a human nasopharyngeal cancer cell line). However, our results raised the possibility that direct physical interactions of folic acid with the surface of the liposomes without incorporating any spacer might prove to be as useful for increasing the uptake of drug-loaded liposomes. The coupling of folic acid onto the liposome surface, according to the procedure used during preparation, suggested that folic acid may form weaker bonds than covalent bonds with the reactive groups of the phosphatidylcholine (i.e. the polar head). It should also be noted that the method of preparation for the folic acid-coupled liposomes was simple compared with other methodologies reported in the literature, in which the links were obtained through chemical conjugation involving many steps in the synthesis.[8,13,14]

The in-vitro evaluation revealed that the particle size and the entrapment efficiency of liposomes were not affected by physical conjugation of folic acid (Table 2). This might suggest that the folic acid molecules were only adsorbed at the surface or at most penetrated the liposome surface superficially and without involving any chemical interaction or competition that would lead to an alteration of the chemical characteristics of the liposomes.

As for the in-vivo study, the bioavailability of cefotaxime was found to increase 1.4–2.0-fold when administered via folic acid-coupled liposomes as compared with folic acid-free liposomes. A higher increase in bioavailability was reported by Anderson *et al.*[25] using vancomycin-loaded liposomes coated with folic acid-poly (ethylene oxide)-cholesterol construct at its surface, in which a 3.2-fold increase in bioavailability was observed for the folic acid-coated liposomes as compared with uncoated ones. The higher increase in bioavailability observed by Anderson *et al.*[25] with vancomycin could have been due to the higher entrapment efficiency achieved with vancomycin (32%) as compared with cefotaxime (22.1%) in this study. In addition, the presence of free folic acid in the preparation used in this study might have also competed with the folic acid-coupled liposomes for binding to folate receptors, thus limiting the uptake of the folic acid-coupled liposomes and hence cefotaxime.

Folate-mediated uptake of macromolecular conjugates, microparticulates and liposomes have been reported by other workers.[8,10,13–16,26] During receptor-mediated endocytosis, the binding of ligand to receptor triggers the complex to be endocytosed from the cell surface. The endocytosed material will then be processed in several ways. Most ligands are dissociated from their receptors by the low pH encountered within the endosomes. The receptors may either be recycled to the cell surface or degraded while the ligand is routed to the lysosomes. Alternatively, the receptor–ligand complex may be unaffected and directly sorted to lysosomes for degradation. In addition, the receptor–ligand complex may be endocytosed and transported to endosomes via coated vesicles. In this case, the endocytosed material escapes the lysosomal attack and is eventually released from the cell by exocytosis.[27] Thus, a particulate drug carrier taken up via receptor-mediated endocytosis could potentially transcytosise the intestinal epithelial cell, resulting in an increase in the oral bioavailability of an otherwise poorly-absorbed drug, which in this study was represented by cefotaxime.

Conclusions

Drug-loaded liposomes were amenable to receptor-mediated endocytosis for further enhancement of the oral bioavailability of encapsulated peptidomimetic drugs. Folic acid was found to be a suitable targeting agent for this purpose. Moreover, it was not necessary for the folic acid to be covalently conjugated to the liposomes.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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