OPTIMIZATION AND IN VITRO EVALUATION OF 5-FLUOROURACIL – LOADED LONG – CIRCULATING LIPOSOMES

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Abstract

5-Fluorouracil (5-FU) is an anticancer drug widely used in the treatment of colorectal cancers. In this work, long-circulating liposomes (LCL) were proposed as carriers able to improve the therapy with 5-FU. The objective was to optimize the formulation of 5-FU-loaded long circulating liposomes (LCL-5-FU) using the method of experimental design and to evaluate the in vitro drug release and cytotoxicity on C26 murine colon carcinoma cells cultivated in monolayer as well as in co-culture with murine peritoneal macrophages. The influence of phospholipids concentration and phospholipids to cholesterol molar ratio was studied on 5-FU liposomal concentration, entrapment efficiency and liposomes’ size. The optimized formulation (LCL-5-FU-OPT) had liposomal 5-FU concentration of 331.4 µg/mL, entrapment efficiency of 3.18 % and liposomes’ size of 200 nm. The in vitro release test has shown a diffusion of 5-FU of 90% in 3 hours. The cytotoxicity data indicated that LCL-5-FU-OPT exerted strong and similar inhibitory effects on the proliferation of C26 tumour cells under both culture conditions as those exerted by free 5-FU administration. In conclusion, the liposomal 5-FU formulation optimization within our studies might offer promise for future anti-cancer therapies based on passive tumour targeting by using LCL.

Rezumat

5-Fluorouracilul (5-FU) este o substanță citostatică utilizată pe scară largă în tratamentul cancerului colorectal. În această lucrare, lipozomi cu timp de circulație prelungit (LCL) au fost propuși ca transportori capabili să îmbunătățească terapia cu 5-FU. Obiectivul lucrării a fost de a optimiza formularea unor lipozomi cu timp de circulație prelungit încărcăcuți cu 5-FU (LCL-5-FU), folosind metoda planurilor experimentale și de a evalua cedarea in vitro a substanței medicamentoase și citotoxicitatea pe celule murine de carcinoom colonic C26, cultivate în monocultură, precum și în co-cultură cu macrofage peritoneale. A fost studiată influența concentrației fosfolipidelor și a raportului molar colesterol:fosfolipide asupra concentrației 5-FU în lipozomi, eficienței încorporării și mărimii lipozomilor. Formula optimizată (LCL-5-FU-OPT) a avut o concentrație în 5-FU de 331,4 µg/mL, eficiența încorporării de 3,18% și mărimea lipozomilor de 200 nm. Testul de cedare in vitro a arătat o difuzie a 5-FU în proporție de 90% în 3 ore. Datele de citotoxicitate au indicat că LCL-5-FU-OPT are efecte inhibitoare puternice și similare, asupra proliferării celulelor tumorale C26, în ambele tipuri de culturi, cu cele exercitate de 5-FU administrat în stare liberă. În concluzie, formularea de lipozomi cu 5-FU optimizată în cadrul acestui studiu ar putea oferi o perspectivă pentru viitoarele terapii antineoplazice bazate pe țintirea tumorală pasivă cu ajutorul LCL.

Keywords: 5-Fluorouracil; long-circulating liposomes; experimental design; optimization; cytotoxicity

Introduction

Anticancer agent 5-fluorouracil (5-FU) is widely used in the treatment of solid tumours such as colorectal cancers. As mechanism, 5-FU induces cytotoxicity mainly through the inhibition of synthesis of DNA and RNA in proliferating cells. However, its clinical utilization is accompanied by several difficulties. Thus, the oral bioavailability of 5-FU is unpredictable due to high variability in enzymatic degradation. Less than 20% of an injected dose undergoes enzymatic activation and the rest is quickly catabolized to inactive metabolites, which leads to a short circulation time in vivo (t½ is about 10 - 20 min), low drug accumulation in tumour and also low antitumor efficacy [26]. Over the years, a better understanding of its mechanism of action has led to the development of several strategies for increasing the anti-cancer activity of 5-FU: to decrease its rapid metabolism, to increase its conversion into active metabolites and to maintain high concentrations in both blood and
tumour over long periods of time [7]. Thus, several approaches have been developed such as the use of prodrugs, bio-modulation and drug delivery systems (liposomes/polymer-nanoparticles). A successful approach has been the development of a pro-drug where the parent drug (capcitabine) is enzymatically converted to the active metabolite in the target tissues. Despite these efforts there is a continued rationale for development of 5-FU formulations with the purpose to extend plasma circulation time and to enhance therapeutic activity of this anti-cancer agent [12, 13, 18, 19, 21-23]. To this end, long-circulating liposomes (LCL) as drug delivery systems for 5-FU could prolong blood residence time of the drug and finally increase 5-FU levels at the sites of the malignancy. One of the most exploited strategies to obtain LCL is based on steric stabilization of the liposome surface with hydrophilic polymers such as polyethylene glycol (PEG). Therefore, these PEG-coated liposomes are also referred to as "stealth liposomes" due to the highly hydrated groups of PEG that inhibit both hydrophobic and electrostatic interactions of blood components at the liposome surface thereby avoiding or retarding opsonisation of liposomes by the cells of reticulo-endothelial system (RES) [24]. Upon administration, stealth liposomes accumulate in tumour regions through a phenomenon known as the enhanced permeation and retention effect (EPR), due to the exacerbated permeability of the tumour vasculature endothelium compared to healthy endothelium [12, 25].

Nevertheless the activity of liposomes as carriers for drugs depends upon various factors such as charge, rigidity, size, composition of the liposomal membrane, drug concentration, stability, release rate and body distribution after administration [16, 20]. One of the most important goals in liposomes formulation is to encapsulate a sufficient amount of therapeutic agent and thus to obtain therapeutically effective liposomes. Encapsulation of molecules such as 5-FU is difficult because it is highly water soluble and it has a small and very permeable molecule [23]. In previous works, 5-FU encapsulation efficiency was evaluated only in conventional liposomes [13, 18].

The aim of this study was to optimize the formulation of long-circulating liposomes containing 5-FU (LCL-5-FU) prepared by the lipid film hydration method. An experimental design has been used with the purpose of evaluating the influence of two formulation parameters, i.e. phospholipids concentration and phospholipids to cholesterol molar ratio, on 5-FU liposomal concentration, encapsulation efficiency and liposomal size. Secondly, the experimental design was used to optimize the preparation formula in order to obtain maximum drug content and encapsulation efficiency at desired liposomal size. Furthermore, this study offers preliminary information regarding the in vitro cytotoxicity of the optimized LCL-5-FU versus free 5-FU on C26 murine colon carcinoma cells cultivated in monoculture as well as in co-culture with murine peritoneal macrophages.

**Materials and Methods**

**Materials.** 5-FU was purchased from Haiers Pharmaceutical, China. Phospholipids (PHL), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), N-(Carboxyl-methoxypolyethyenglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (Na-salt) (MPEG-2000-DSPE), from Lipoid GmbH (Germany); cholesterol (CHOL) from sheep wool (> 92.5%, GC) from Sigma-Aldrich (Germany). All the other solvents and reagents were of analytic grade purity, commercially available. C26 murine colon carcinoma cell line was provided by CLS Cell Lines Service GmbH, Germany.

**Preparation of LCL-5-FU.** The liposomes were prepared by lipid film hydration method, essentially according to the technique first described by Bangham et al. [3]. Briefly, DPPC, MPEG-2000-DSPE molar ratio 9:5:0.5 and CHOL were dissolved in ethanol in a round-bottomed flask. After dissolution, the solvent was evaporated under reduced pressure at 45°C in a rotary evaporator, leading to the formation of a thin and homogeneous film of lipids on the surface of the flask. In order to completely remove the residual solvent, the film was maintained under a nitrogen gas flow for 1 h. Then the film of lipids was hydrated with 5 mL of 80 mM 5-FU aqueous solution in phosphate buffered saline (PBS, pH = 7.4) for 15 minutes at 45°C. Liposomes’ size was reduced by multiple extrusion steps (High Pressure Homogenizer LiposFast LF-50, Avestin Europe GmbH, Germany) through polycarbonate membranes with a final pore size of 200 nm. Unencapsulated 5-FU was removed by dialysis in a Slide-A-Lyzer cassette with a molecular weight cut-off of 10 kDa with repeated changes of buffer [2]. Finally, LCL-5-FU was stored at 4°C until analysis.

**Experimental design.** In order to optimize the preparation formula of LCL-5-FU, an experimental design with 2 factors and 3 levels was developed using Modde 10 software (Umetrics, Sweden) [10]. The selected formulation factors (independent variables) were: the PHL molar concentration in initial dispersion (X₁) and the PHL:CHOL molar ratio (X₂) (Table I). The dependent variables of the experimental design (liposomal properties) were: 5-FU concentration in final liposomal dispersion (µg/mL) (Y₁), liposomes size (nm) (Y₂), polydispersity index (PDI) (Y₃) and encapsulation efficiency (%) (Y₄). The matrix of experimental design is presented in Table II.
The statistical correlation between the formulation factors studied and the observed responses and the statistical parameters calculations were performed with the statistical module from Modde 10 software. Partial Least Squares (PLS) method was used for data fitting and for calculation of statistical parameters. The equation used for determination of the regression coefficients of each investigated factor was the following:

\[ Y_n = b_0 + b_1X_1 - b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2 \] (1)

Where: \( Y_n \) is the dependent variable; \( b_0 \) is the model constant; \( b_1 \), \( b_2 \), shows the variables of related variables on the response; \( b_{11}, b_{22} \) are quadratic coefficients and shows a nonlinear relationship; \( b_{12} \) are interaction coefficients between the studied factors [1, 10].

The validity of the experimental design was checked by determination of the following statistical parameters: \( R^2 \) (representing the variation fraction of the response explained by the model) and \( Q^2 \) (the variation of the response that can be predicted by the model). Both \( R^2 \) and \( Q^2 \) are numbers, usually between 0 and 1. Values close to 1 for both \( R^2 \) and \( Q^2 \) indicate a very good model with excellent predictive power. \( R^2 \) and \( Q^2 \) provide the best summary of fitting the model. \( R^2 \) represents over-estimated and \( Q^2 \) underestimated measures, respectively, of the quality of fit of the model [1, 10]. The analysis of variance (ANOVA) test was also performed. This test shows if the variance of the results is determined by the modifications of the formulation factors or represents a variance determined by experimental errors [20].

**Determination of particle size and Pdl.** Mean particle size and Pdl were determined by dynamic light scattering method (Zetasizer Nano ZS, Malvern Instruments, UK). Liposomes samples were adequately diluted with ultra-pure water and analysed at 25°C, with a scattering angle of 90°. The dynamic light scattering data was collected using a helium laser as the light source and mean results were provided by photon correlation spectroscopy.

**Determination of 5-FU content and encapsulation efficiency.** The samples were prepared by liposomes dissolution in methanol and dilution in water. Analyses were performed using a HPLC-UV method on an Agilent 1100 Series chromatograph (Agilent Technologies, USA), equipped with a Zorbax SB-C18 column (100 mm x 3 mm i.d., pore size 3.5 µm, Agilent Technologies), protected with an online filter. Chromatographic separation was done at 35°C. The mobile phase, 100% phosphoric acid 0.1%, was delivered at a flow rate of 1 mL/min. 5-FU was quantified by UV detection at \( \lambda = 266 \) nm [19]. Liposomal 5-FU was expressed both as concentration (µg/mL) and encapsulation efficiency (EE, %). The encapsulation efficiency was expressed as a percentage of entrapped drug and was calculated using the equation:

\[ EE(\%) = \frac{\text{Entrapped 5-FU}}{\text{Total 5-FU}} \times 100 \] (2)

**Optimization of LCL-5-FU formulation.** The optimization was performed using desirability function, \( f(ds) \), that searches for the best possible combination of factor settings that predicts a result inside the response specifications and as close as possible to the targets [10]. The optimum formulation of 5-FU-loaded liposomes (LCL-5-FU-OPT) was selected based on the criteria of maximizing liposomal 5-FU concentration and the entrapment efficiency for vesicles size up to 200 nm.

**In vitro drug release.** Drug release from the liposomes was carried out by the dialysis method using Slide-A-Lyzer cassettes with a molecular weight cut-off of 10 kDa (Thermo Scientific). Liposomes were dialyzed against PBS pH = 7.4 as receptor phase, at 37°C, 80 rpm. At predetermined time intervals samples were removed and replaced.
with buffer. The released 5-FU was quantified using the HPLC-UV method mentioned above. All in vitro release studies were run in triplicate and mean values were reported.

**Cell line and culture conditions.** C26 murine colon carcinoma cells (CLS Cell Lines Service GmbH) were cultured in RPMI-1640 medium (Lonza), supplemented with 10% heat-inactivated fetal bovine serum, (Lonza) as monolayer at 37°C in a 5% CO₂ humidified atmosphere.

**Preparation of macrophages:** C26 murine colon carcinoma cells co-cultures. Culture of inflammatory macrophages were established from peritoneal exudates collected by lavage from 6-8 week-old Balb/c mice that had been injected intraperitoneally with 1 mL of 3% thioglycollate broth 3 days before as reported previously [5]. Co-cultures were prepared by seeding C26 tumour cell suspensions on macrophage monolayers. In our experiments, we used macrophages: tumour cell co-cultures at a 1:4 cell density ratio. Previous studies have proved that this cell density ratio ensures the optimal cytokine interplay between tumour cells and macrophages that provides an approximation of physiological conditions of colon carcinoma development in vivo [15].

**Cell proliferation assay.** To assess the effects of LCL-5-FU-OPT versus free 5-FU on the cell proliferation, C26 colon carcinoma cells were plated into 96-well plates at a concentration of 10,000 tumour cells/well for 24 h. The effects of different treatments were tested on C26 cell monoculture as well as on the co-culture of C26 cells with macrophages as indicated above. As controls for liposomal lipids toxicity, C26 cells in both culture conditions were incubated with empty liposomes (*i.e.* devoid of drug) at similar concentrations as those used for the preparation of working concentrations of LCL-5-FU-OPT. Cells cultivated only in medium were used as controls. After 72 h of incubation with LCL-5-FU-OPT and free 5-FU respectively, the proliferative activity of the C26 cells was tested by using ELISA BrdU-colorimetric immunoassay (Roche Applied Science, Penzberg, Germany) according to the manufacturer’s instructions [6, 14].

This method is based on the incorporation of the pyridine analogue – bromodeoxyuridine (BrdU) – instead of thymidine into the DNA of proliferating cells. C26 colon carcinoma cells were incubated with BrdU solution for 24 h and the culture medium was completely removed from each well. Following this step, the cells were fixed and the DNA was denatured. A monoclonal antibody conjugated with peroxidase – anti-BrdU-POD – was added in each well, in order to detect the incorporated BrdU in the newly synthesized cellular DNA. The antibody was removed after 1 h incubation, and the cells were washed three times with phosphate buffered saline. A peroxidase substrate (tetramethyl-benzidine) was added in each well, and the immune complexes were detected by measuring the absorbance of the reaction product at 450 nm, with a reference wavelength of 655 nm. Data from different experiments were reported as mean ± standard deviation (SD) of 3 independent measurements.

**Results and Discussion**

The experimental design used within this work allowed the evaluation of the influence of formulation parameters on LCL-5-FU properties with the ultimate aim to optimize a liposomal 5-FU formulation for systemic therapy of colorectal cancer. Our study was focused on the in vitro optimization of a PEG-ylated liposomal encapsulated 5-FU formulation designed for the passive colon cancer targeting of 5-FU after intravenous administration. Several formulation parameters that can influence liposomes properties have been reported in the literature: method of manufacture, nature and concentration of PHL, PHL:CHOL ratio, drug nature (hydrophobic/hydrophilic molecule), drug concentration, PHL:drug ratio [18, 20, 21]. Among these, we have chosen to evaluate the PHL concentration (20, 40 and 60 mM) and the PHL:CHOL molar ratio (5:1, 10:1 and 20:1). It is known that PHL concentration plays an important role in the circulating lifetime of liposomal carriers due to a decreased phagocytic capacity of macrophages after the ingestion of high lipid doses or to a saturation of plasma factors that bind to circulating liposomes and results in their opsonisation [9]. The incorporation of cholesterol into liposomes can increase their stability and reduces membrane fluidity and permeability [17]. A high fraction of cholesterol has been recognized to contribute to long-circulating properties of liposomes [11]. However, at low cholesterol levels, the membrane is very fluid and tends to release the entrapped drug. Thus, the optimum cholesterol concentration should be determined to achieve good encapsulation efficiency for the drug and to prevent the rapid release of the entrapped drug.

5-FU was encapsulated into LCL (PEG-ylated liposomes) by film hydration method and the influence of two formulation parameters (PHL molar concentrations and PHL to CHOL molar ratios) on liposomes characteristics was studied according to an experimental design consisting of eleven formulations. The PHL mixture used for preparation of all formulations in the experimental design was represented by a combination of DPPC and MPEG-2000-DSPE (molar ratio 9.5:0.5). Regarding the formulation parameter drug concentration, all the formulations were prepared...
using a single 5-FU concentration, i.e. 80 mM in PBS pH = 7.4, a high concentration considering that the solubility of 5-FU in aqueous medium is about 100 mM [4]. The selection of the 5-FU concentration was based on the fact that for hydrophilic molecules as 5-FU, the higher the initial drug concentration in hydration solution the greater the liposomal concentration. The resulting liposomes were evaluated regarding 5-FU content (µg/mL), size (mean diameter; nm), size distribution (PdI) and encapsulation efficiency (%). The experimental results are presented in Table III.

Table III

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Y1 (µg/mL)</th>
<th>Y2 (nm)</th>
<th>Y3 (%)</th>
<th>Y4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>5.6</td>
<td>192</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td>N2</td>
<td>21.1</td>
<td>206</td>
<td>0.08</td>
<td>0.20</td>
</tr>
<tr>
<td>N3</td>
<td>56.3</td>
<td>208</td>
<td>0.07</td>
<td>0.54</td>
</tr>
<tr>
<td>N4</td>
<td>43.5</td>
<td>187</td>
<td>0.09</td>
<td>0.42</td>
</tr>
<tr>
<td>N5</td>
<td>35.6</td>
<td>184</td>
<td>0.08</td>
<td>0.34</td>
</tr>
<tr>
<td>N6</td>
<td>212.4</td>
<td>200</td>
<td>0.07</td>
<td>2.04</td>
</tr>
<tr>
<td>N7</td>
<td>66.1</td>
<td>182</td>
<td>0.09</td>
<td>0.63</td>
</tr>
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<td>N8</td>
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<td>0.10</td>
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<td>N9</td>
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<td>221</td>
<td>0.13</td>
<td>5.19</td>
</tr>
<tr>
<td>N10</td>
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<td>192</td>
<td>0.10</td>
<td>0.81</td>
</tr>
<tr>
<td>N11</td>
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<td>200</td>
<td>0.12</td>
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</tbody>
</table>

Y1, 5-FU content (µg/mL); Y2, liposomal size (nm); Y3, polydispersity index; Y4, encapsulation efficiency (%)

Experimental design analysis. Goodness of fit. The results obtained after fitting the experimental data with the experimental design, using the statistical module from Modde 10 software, i.e. the values of the calculated statistical parameters, are shown in Figure 1.

![Figure 1](image)

Summary of fit for the experimental design
Y1, 5-FU content (µg/mL); Y2, liposomal size (nm); Y3, polydispersity index; Y4, encapsulation efficiency (%)

The results fit excellent the responses Y1 and Y4 (R² higher than 0.98, Q² higher than 0.88), and well the Y2 and Y3 responses (R² higher than 0.88, Q² higher than 0.50). The results of the ANOVA test have shown a good correlation between the experimental conditions and the results (a significant influence of the formulation factors on all studied responses), as p for model was lower than 0.05 and p for residual was higher than 0.05. These results confirm the relevance of the defined experimental design.

Experimental design analysis. The influence of formulation parameters on liposomes’ properties. The regression coefficients of the equation used to fit the experimental data with the chosen model show the influence of the studied factors on each response and possible influence of interactions between factors on the responses. The influence of the formulation factors on the responses was presented as scaled and centered regression coefficients and as response surface plot. The influence of the formulation factors on 5-FU concentration in LCL and on the entrapment efficiency is presented in Figure 2 and Figure 3, respectively.
Figure 2.

The influence of formulation factors on 5-FU concentration (µg/mL) in LCL (Y₁)
(A) Scaled and centered regression coefficients and (B) response surface for predicting 5-FU liposomal concentration with respect to PHL concentration (X₁; mM) and PHL:CHOL molar ratio (X₂)

Figure 3.

The influence of formulation factors on encapsulation efficiency (%) (Y₄)
(A) Scaled and centered regression coefficients and (B) response surface for predicting the encapsulation efficiency with respect to PHL concentration (X₁; mM) and PHL:CHOL molar ratio (X₂)

As shown in Table III, liposomal 5-FU concentration varied from 5.6 to 540.5 µg/mL for various combinations of factors. Because concentration of our 5-FU solution used during liposomes preparation was constant (total 5-FU for all formulations was the same), the entrapment efficiency was proportionally with the final liposomal concentration of 5-FU and ranged from 0.06 to 5.19%. According to Figure 2, 5-FU concentration in final liposomal dispersion and consequently the encapsulation efficiency (Figure 3) were significantly influenced by the two formulation factors, the PHL concentration and PHL:CHOL molar ratio. Both PHL concentration and PHL:CHOL molar ratio had a positive influence on liposomal 5-FU concentration and on encapsulation efficiency. Thus, the increase of phospholipids concentration and the decrease of the CHOL amount in the lipid phase (PHL:CHOL molar ratio from 5:1, 10:1 to 20:1) will result in higher 5-FU concentration in LCL and higher entrapment efficiency. A significant interaction between formulation factors (X₁ and X₂) can also be observed on both liposomal drug concentration and on its encapsulation efficiency. Therefore, the increase of these responses with reduction of cholesterol content in liposomes is more important for higher phospholipids concentrations. The influence of the formulation factors on liposomes size and liposomes’ size distribution expressed by Pdl is presented in Figure 4 and Figure 5 respectively.
The mean diameter of the eleven liposomal formulations was between 184 and 221 nm (Table III). This domain size was determined by the porosity of the polycarbonate membrane used for the extrusion, which corresponded to 200 nm. In the conditions of the experimental design, liposomes size varied slightly and therefore the two analysed formulation parameters had a little influence on liposomes size. However, as shown in Figure 4, LCL size was significantly influenced by the PHL concentration, the increasing of this parameter determining a slight increase of the liposome size. Although X2 does not have a significant influence on liposomal size, its influence on this response is nonlinear, as illustrated by the quadratic coefficient X2*X2. As shown in Table III, the liposomes size homogeneity is good, the values of PdI ranging between 0.07 and 0.13. It can be observed that the PdI is significantly influenced by the interaction between PHL concentration and the PHL:CHOL molar ratio (interaction X1*X2). Therefore, the highest PdI values are obtained for high PHL and low CHOL content (high PHL:CHOL molar ratio) (Figure 5).

Phospholipids concentration had a significant influence on the responses. Thus, liposomal size, liposomal drug concentration and encapsulation efficiency were positively influenced by this formulation factor, meaning that increasing PHL concentration determines the increase of liposomal size, 5-FU concentration and EE. High PHL concentrations determine an increase of the viscosity of liposomal dispersion and increased stability of the vesicles to disruption in the homogenization step of the preparation process. This finding might explain the direct dependence between size and PHL concentration (Figure 4).

In accordance with previous studies focused on conventional liposomes encapsulating 5-FU prepared via passive encapsulation method, the encapsulation efficiency of 5-FU was low, between 0.06 and 5.19% (Table III), and dependent on PHL concentration [13, 18, 21]. It is known that the increase of lipid concentration level leads to an increase in the number of liposomes per millilitre of liposomal dispersion [27], thus explaining the improvement of liposomal 5-FU concentration and encapsulation efficiency for higher PHL concentration.
In vitro drug release. The liposomal formulations were tested with different PHL:CHOL molar ratios: 20:1, 10:1 and 5:1 (in all formulations the 5-FU concentration was the same). As can be seen, 5-FU was released from liposomes rapidly, about 90% of the encapsulated drug being released within 3 hours, and the concentration of CHOL in the PHL bilayer had not a significant influence on the release rate.

Figure 6. In vitro 5-FU release from LCL with different PHL:CHOL molar ratios

It is known that drug release from liposomes may be influenced by several factors, one of the most important being the composition of the liposomal bilayer. Thus, the presence of CHOL led to a bilayer more rigid and less permeable for drugs, determining the decrease of release rate (this effect increases with the increasing of CHOL amount) [13, 17, 21]. Our results did not indicate a significant difference between the three CHOL concentrations (Figure 6). A possible explanation for this result might be that the tested concentrations were too small to have a high impact on the release properties of LCL. Unfortunately, increasing the CHOL proportion has a negative effect on 5-FU loading in LCL and this limits the use of high CHOL concentrations (the PHL:CHOL molar ratio in the optimized formulation being of 15:1), this observation being in agreement with the results of other authors [21].

In vitro cytotoxicity of the LCL-5-FU-OPT. In addition to the studies related to the in vitro optimization of LCL formulation we tested the anti-proliferative effects of the LCL-5-FU-OPT. The effects of different treatments on cell proliferation were expressed as percentage of inhibition compared to the proliferation of the controls and shown in Figure 7 A and B. C26 murine colon carcinoma cells cultivated alone as well as in co-culture with peritoneal macrophages were incubated for 72 h, with increasing 5-FU micromolar concentrations ranging from 0.5 to 8 µM administered as LCL-5-FU-OPT and free 5-FU. Untreated cells were used as controls. After incubation of C26 cells in both culture conditions with empty LCL only minor cytotoxic effects (lower than 10% compared to the...
controls) induced by liposomal lipids were noted (Figure 7 A and B). The proliferation of C26 tumour cells was strongly affected (higher than 50% compared to the proliferation of the untreated cells) at concentrations ranging from 2 to 8 µM of 5-FU administered as liposomal as well as free formulations under both culture conditions (Figure 7 A and B). No influence of macrophages on the cytotoxicity of both LCL-5-FU-OPT and free 5-FU on C26 colon carcinoma cells was noted since the antiproliferative effects were similar under both culture conditions (Figure 7 A and B).

Since previous studies have shown that tumour-associated macrophages (TAM) could modulate the effects of various cytotoxic drugs by either enhancing chemo-sensitivity or inducing chemoresistance in different tumour models [8], we tested the cytotoxicity of LCL-5-FU-OPT versus free 5-FU on C26 colon carcinoma cells co-cultivated with peritoneal macrophages at a cell density ratio that can provide an approximation of physiological conditions of colon carcinoma development in vivo [15]. Our preliminary results pointed out those LCL-5-FU-OPT exerted strong inhibitory effects on the proliferation of C26 tumour cells in monocyte as well as after their co-cultivation with macrophages. Moreover the presence of macrophages did not influence the cytotoxicity of 5-FU after encapsulation in LCL as the anti-proliferative effects of LCL-5-FU-OPT were similar under both culture conditions (Figure 7 A and B). Although at the 5-FU concentrations tested we did not see any additional inhibitory effects of LCL-5-FU-OPT on C26 cell proliferation compared to those exerted by free 5-FU, the tumour-targeting capability of LCL will enable in vivo site-specific delivery of 5-FU increasing the intra-tumourial drug concentration and thereby will intensify the inhibitory effects of 5-FU.

Conclusions

In conclusion, in this work we optimized the preparation of long-circulating liposomes containing 5-FU by lipid film hydration method. With the help of an experimental design we evidenced that the PHL concentration and the PHL:CHOL molar ratio significantly influenced the 5-FU liposomal properties (5-FU concentration, entrapment efficiency and liposomes’ size). High drug content and encapsulation efficiency and a liposomal size below 200 nm were established as the desired characteristics of the optimum formulation. These requirements have been met when using high PHL concentration and low CHOL content. However, the 5-FU encapsulation efficiency was low (around 3%), being in line with the results reported in literature for hydrophilic drugs. The encapsulated drug is rapidly released in vitro from the formulated LCL, irrespective of the PHL:CHOL molar ratio. Moreover, our preliminary studies suggested that LCL-5-FU-OPT exerted strong inhibitory effects on the proliferation of C26 tumour cells irrespective of the presence of macrophages, offering promise for future anti-cancer therapies based on this formulation.

Declaration of interest

The authors report no conflicts of interest.

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