Published in final edited form as:

Anticancer Agents Med Chem. 2017 ; 17(2): 301–308.

Cetuximab Immunoliposomes enhance Delivery of 5-FU to Skin Squamous Carcinoma Cells

Raquel Petrilli^{1,2}, Josimar O. Eloy^{1,2}, Renata F. V. Lopez¹, and Robert J. Lee^{2,*}

¹School of Pharmaceutical Sciences of Ribeirao Preto, University of Sao Paulo, Av. Cafe s/n, 14040-903, Ribeirao Preto, SP, Brazil

²College of Pharmacy, The Ohio State University, Columbus, 500 W 12th Ave, Columbus, OH 43210, USA

Abstract

Topical chemotherapy of skin cancers is a promising strategy for reduction of side effects and for improvement of patient compliance. The combination of the chemotherapeutic 5-fluoruracil (5-FU) and the anti-EGFR antibody cetuximab is a strategy to inhibit tumor growth. Their skin penetration, however, is hampered by their high hydrophilicity, which could be improved by encapsulation in delivery systems. Furthermore, it is a challenge to encapsulate hydrophilic drugs. The conjugation of an antibody to a liposome, maintaining its activity, is also a difficult task. Thus, we aimed to develop 5-FU liposomes and cetuximab-conjugated liposomes (immunoliposomes) of 5-FU to improve drug cytotoxicity against skin cancer cells. We characterized them by particle size, zeta potential, loading efficiency and antibody integrity. To optimize the loading efficiency of 5-FU, a series of liposomes were prepared, using different methods and drug-to-lipid ratios. Liposomes containing DSPC and Chol at drug-to-lipid ratio 0.1 prepared by the thin lipid hydration method resulted in the best 5-FU encapsulation and were chosen to conjugate with cetuximab. Cetuximab was directly coupled to preformed liposomes using DSPE-mPEG2000-Mal as an anchor. In A431 skin carcinoma cells, at 72 h, 5-FU liposomes showed a 5-fold lower IC₅₀ than 5-FU solution. Additionally, 5-FU immunoliposomes resulted in a 4-fold lower cetuximab IC₅₀ than cetuximab solution, demonstrating synergism with a combination index lower than 1 and potential to improve 5-FU and cetuximab cytotoxicity.

Keywords

5-FU; cetuximab; immunoliposomes; skin delivery; cytotoxicity; squamous cell carcinoma

1. INTRODUCTION

Squamous cell carcinoma (SCC) is a malignant tumor from epithelial origin, derived mainly from squamous cells. As these cells represent a major part of epidermis, SCC is the second

*Address correspondence to this author at the College of Pharmacy, The Ohio State University, Columbus, 500 W 12th Ave, Columbus, OH 43210, USA; Tel: (614) 292-4172; lee.1339@osu.edu.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

most frequent non-melanoma skin cancer type [1]. The topical and localized administration of drugs or drug carriers for SCC treatment, mainly for the skin, is a promising alternative to reduce the side effects caused by systemic chemotherapy and increase patient compliance for areas or aged patients, in which surgery is not desirable [2]. Current topical treatments of skin cancer include semi-solid formulations of 5-fluorouracil (5-FU), diclofenac and imiquimod.

5-FU has been considered the topical treatment of choice for actinic keratosis since its approval in 1970 by the US FDA [3] and it is indicated for the treatment of basal cell carcinoma [4]. For SCC treatment, although potentially promising results have been shown [5], the relative long treatment period and partial inefficacy in the deep layers of skin limit its uses. Moreover, side effects, such as an intense local inflammatory reaction, result in a lack of patient compliance. Thus, due to the low penetration of hydrophilic drugs like 5-FU into the skin, different approaches can be used such as microneedles [6] or drug delivery systems, as proposed herein. Encapsulation of the drug in liposomes could improve drug delivery to the tumor and reduce local side effects [7, 8]. However, 5-FU trapping efficiency in liposomes is reported to be only 2–6%, depending on the lipid composition [9, 10].

The efficacy of 5-FU topical treatment against SCC can also be improved by its combined administration with cetuximab, an IgG1 recombinant human/mouse chimeric antibody that blocks EGFR activation and phosphorylation induced by other ligands [11]. Nevertheless, EGFR is expressed by some normal cells, but is generally overexpressed in cancer, such as SCC, as a result of hyper-regulation, amplification, mutation or and increased expression of ligands and co-factors, which cause a malignant effect for cancers [12]. Therefore, inhibition of EGFR by cetuximab is a useful strategy that has been used to inhibit tumor growth [13]. The combination of cetuximab and 5-FU has been reported with acceptable risk-to-benefit profile for the intravenous treatment of SCC of the head and neck [14]. However, for a topical SCC skin treatment, cetuximab and 5-FU need to penetrate deep into the skin to reach tumor cells and to be effective. The hydrophilicity of 5-FU and the high molecular weight of cetuximab (MW = 145781.6 g/mol) certainly hamper their skin penetration and, consequently, the tumor treatment efficacy. Nevertheless, the use of antibodies can be useful due to their own therapeutic efficacy either alone or in combination therapy. Additionally, immunoliposomes when using subtherapeutic doses of the antibody can act as drug targeting agents for tumor cells expressing the antibody receptor [15, 16].

Cetuximab conjugation to the surface of liposomes, generating immunoliposomes, is a promising strategy to carry both of the drugs through the first skin barrier, the stratum corneum, and also to target the cancer cells in high concentrations. Therefore, the aim of this study is to prepare cetuximab-immunoliposomes containing 5-FU to improve drugs cytotoxicity against SCC for a further topical administration. In this study we have developed 5-FU liposomes and immunoliposomes with cetuximab, which, to our knowledge, have never been reported. For the development of liposomes, different strategies were used as an attempt to increase 5-FU loading efficiency. Cetuximab was covalently attached to the surface of liposomes by direct coupling. The liposomes and immunoliposomes were characterized and the cytotoxicity and combination index of 5-FU and cetuximab in liposomes were assessed *in vitro* using the SCC EGFR positive A431 cell line.

2. MATERIALS AND METHODS

2.1. Materials

L- α -Phosphatidylcholine from soybean (SPC), 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Avanti polar lipids (Alabaster, AL, USA). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N[maleimide(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG-Mal) was obtained from LaySan Bio (Arab, AL, USA). Erbitux 2 mg/mL (Cetuximab) was a kind gift from cancer hospital clinical pharmacy. 5-Fluorouracil (5-FU), Traut's reagent and 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Precision Plus Protein Dual Color Standards, Ready gel Tris-HCL precast gels, Laemmli sample buffer 4X, 2-mercaptoethanol and Tris/Glycine/ SDS buffer 10X were purchased from Biorad (Hercules, CA, USA) and used according to manufacturer's instructions. Sepharose CL-4B and disposable PD-10 desalting columns were obtained from GE Healthcare (Pittsburgh, PA, USA). Antibiotic/antimycotic solutions, fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Grand Island, NY). Bicinchoninic acid protein assay (BCA) kit was obtained from Thermo Scientific (Pittsburg, PA, USA). A431 cells (ATCC® CRL1555™) were purchased from American Type Culture Collection (ATCC) and cultivated according to the instructions.

2.2. Development and Preparation of 5-FU Liposomes

For 5-FU quantification in liposomal formulations, a spectrophotometric assay was developed and validated. A stock standard solution of 1.3 mg/mL in water was prepared and serial dilutions were done. The solutions were assessed for absorbance in 265 nm. The analyses were carried out using distilled water as blank. For method validation, the selectivity, linearity, precision, accuracy, limits of detection and quantification were investigated following the ICH Harmonized Tripartite Guideline.

To optimize the loading efficiency of 5-FU, different lipids (SPC, DPPC and DSPC) were combined to Chol using the molar ratio at 55:45 lipid:Chol (100 μ mol total lipids) and drug-to-lipid ratio at 0.2, based on the previous study by THOMAS *et al.* [17]. The liposomes were first prepared using the classic thin-film lipid hydration method [18] and freeze-thaw cycles. Briefly, appropriate amounts of the lipids were placed in a round-bottom flask and dissolved in chloroform until a clear solution was obtained. The flask was attached to the rotary evaporator and kept under vacuum and immersed in a water bath set at 60°C with 100 rpm rotation until formation of a dry lipid film (30 min). Phosphate buffer (PBS 7.4) containing the drug was added to the flask and the rotation continued until complete film hydration (1 h). Then, liposomes were subjected to 5 freeze/thaw cycles by alternately placing the sample vial in a dry ice bath and warm water bath (37°C) [19, 20]. All liposomes were extruded for 5 times through 0.2 μ m pore size polycarbonate filters, followed by extrusion for 3 times through 0.1 μ m pore size. The DSPC:Chol lipid composition was chosen and the active loading, reverse phase evaporation method and different drug-to-lipid ratios were tested.

The active loading method was based on the study by THOMAS *et al.* [17] with modifications, summarized in Fig. 1. Basically, an entrapment solution was prepared composed of high molecular weight polyethylenimine (MW = 25,000, PEI) and copper acetate (Cu:PEI ratio of 1:4; final copper concentration 600 mmol/mL, 5 mL) and used to hydrate the thin lipid film at 60°C for 1 h. After extrusion, the liposomes were purified in a Sepharose CL-4B column and the fractions containing the liposomes were combined to 5-FU solution. After mixing in a rotary evaporator for 1 h, the liposomes were separated from the free drug using the CL-4B column and the 5-FU loading efficiency was assessed, using the validated method.

Liposomes were also prepared using the reverse phase evaporation method [19] (Fig. 1). First, the lipids were combined to a mixture of 2-propanol/ chloroform/ 5-FU solution (2:1:1) and the emulsification was formed under agitation. The organic solvents were evaporated and the mixture was resuspended in PBS and extruded. Finally, the loading efficiency was evaluated using the spectrophotometric assay.

2.3. Characterization of 5-FU Liposomes

2.3.1. Particle Size and Zeta Potential—The average particle diameters, size distributions and zeta potentials of the liposomes were analyzed using a dynamic light-scattering method (DLS) in a Zetasizer NS 90 (Malvern Instruments) using a 10-mW HeNe laser operating at 633 nm with an incidence detection angle of 90° at 25°C. All of data analysis was performed in automatic mode. The results are given as the mean ± standard deviation (SD) of triplicate analyses.

2.3.2. 5-FU Loading Efficiency—The separation of 5-FU loaded liposomes and the free drug was performed using size exclusion chromatography with a Sepharose CL-4B column (0.7 cm × 10 cm). The fractions were collected using PBS pH 7.4 and the absorbance was verified both in 265 nM for free 5-FU quantification and in 400 nm for liposome detection. The liposomal fractions (4–6) and the free drug (10–14) were separated and the total liposomes (prior to the separation with the Sepharose column) and the free drug were quantified using the validated method. The loading efficiency was determined as the percentage of free 5-FU relative to the total drug amount (Equation 1). Liposomes were lysed using DMSO, vortexed for 2 min and sonicated in an ultrasound bath for 10 min. Finally, the samples were diluted in deionized water prior to the quantification.

$$\% \text{ loading efficiency} = (C_{5\text{-FU Total}} - C_{\text{Free 5-FU}}) / C_{5\text{-FU Total}}, \quad (\text{Equation 1})$$

where $C_{5\text{-FU Total}}$ is the total 5-FU concentration in the liposomes after extrusion and $C_{\text{Free 5-FU}}$ is the concentration of the free drug

2.3.3. Liposomal Stability—The liposomal formulation chosen for the targeting protocol (DSPC:Chol, drug-to-lipid ratio 0.1, prepared by the thin lipid evaporation method) was monitored for stability, regarding particle size, polydispersity, zeta potential and loading efficiency for 30 days at 4°C. The graphs represent the mean ± standard deviation (SD) of

triplicate analyses. The results were subjected to Kruskal-Wallis statistical test followed by the post-test of Dunns.

2.4. Development and Characterization of 5-FU Immunoliposomes

2.4.1. Preparation of Immunoliposomes—The targeting protocol was based on the previously developed method by LAGINHA *et al.* [21]. Briefly, cetuximab (1 mg/mL) was thiolated using Traut's reagent:Cetuximab 40:1 (mol/mol) in PBS/EDTA buffer (5 mM, pH 8.0) for 1 h at 37°C. The unreacted Traut's reagent was removed by chromatography using a disposable PD-10 desalting column collecting 1 mL fractions eluted with PBS/EDTA buffer (5 mM, pH 8.0). Cetuximab concentration was determined by BCA assay following the manufacturer's instructions and the fractions containing the thiolated cetuximab were combined (fractions 4–6). For the antibody conjugation, DSPE-PEG-lipids-Mal was used as an anchor for cetuximab attachment. Stealth non-targeted liposomes were prepared using DSPC:Chol:DSPE-PEG-NH₂ (55:45:2.5) in order to evaluate the effect of DSPE-PEG for 5-FU loading efficiency. The immunoliposomes were prepared using DSPC:Chol:DSPE-PEG-Mal (55:45:2.5) by the thin lipid evaporation method and hydrated in 2 mL PBS (pH 7.4) containing or not 5-FU, followed by extrusion for 5 times through 0.2 µm pore size polycarbonate filters and for 3 times through 0.1 µm pore size. The thiolated cetuximab (fractions 4–6) was combined with the liposomes and the mixture was incubated overnight at room temperature. Unconjugated cetuximab was removed by chromatography using a Sepharose CL-4B column eluted with PBS (pH 7.4). The immunoliposomes were characterized by particle size, polydispersity, zeta potential, 5-FU loading efficiency, targeting efficiency and antibody integrity.

2.4.2. Evaluation of Conjugation Efficiency—The conjugation efficiency was evaluated by the BCA protein assay method, following the manufacturer's instructions. Briefly, the working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). In 96 well microplates, a standard curve (25–750 µg/mL) and test samples were applied using 25 µL of the standard/sample and 200 µL of the working reagent. The microplates were placed for 30 s on a microplate shaker and then incubated at 37°C for 30 min. Finally, the absorbance was measured at 562 nm and the conjugation efficiency was calculated as the percentage of cetuximab conjugated to the liposomes compared to the amount of thiolated cetuximab.

2.4.3. Antibody Integrity—Cetuximab integrity after covalent linkage to liposomes was evaluated by SDS-Page electrophoresis under reducing conditions, based on previous studies by Yang *et al.* [22]. Samples containing 1 mg of protein were previously prepared by the addition of the reducing agent 2-mercaptoethanol and Laemmli sample buffer. The samples were heated at 60°C for 10 min for denaturation prior to gel application. SDS-Page 4–20% mini-PROTEAN gels were loaded with the samples. The electrophoresis was carried out at 175 V for 50 min in Tris/glycine/SDS buffer pH 8.3 and the precision plus dual color protein (Biorad) was used for molecular weight confirmation. The staining was performed using QC colloidal coomassie stain (Biorad), followed by destaining with deionized water in 3 cycles of 30 min each. Finally, the gels were photographed.

2.5. Cytotoxicity Evaluation in A431 Cell Line

2.5.1. Cell Culture—Human squamous cell carcinoma (A431) cells obtained from the American Type Culture Collection (Rockville, MD) cells were cultivated in DMEM, supplemented with 10% heat-inactivated FBS and 1% (v/v) of an antibiotic/antimycotic solution containing 10,000 IU of penicillin, 10 mg of streptomycin and 25 µg of amphotericin B per mL and grown at 37°C in a 5% CO₂ atmosphere.

2.5.2. Cytotoxicity Study—The cytotoxicity induced by the samples was evaluated by the MTT assay. For that, 1×10⁴ cells/well were seeded onto 96-well plates and incubated for 24 h at 37°C in 5% CO₂. The cells were washed and incubated with the samples (5-FU, cetuximab solutions and cetuximab-targeted and non-targeted 5-FU liposomes) diluted in incomplete DMEM medium for 72h and 120h. Then, the cells were washed twice to remove all samples and culture medium/MTT solution 2.5 mg/mL were added prior to incubation for 4 h at 37°C. After this procedure, the medium containing the MTT reagent was discarded and the formazan crystals were solubilized in 200 µL of DMSO. The quantifications were performed by measuring the absorbance values at 570 nm using a microplate reader. The cellular viability was calculated as the percentage of viable cells compared to the control group and half maximum inhibitory concentration (IC₅₀) was calculated. The method of CHOU and TALALAY *et al.* [23] was used to calculate the combination index between the drugs (5-FU and cetuximab), as represented in Equation 2:

$$CI = (D_1/D_{x1}) + (D_2/D_{x2}), \quad (\text{Equation 2})$$

where D₁ and D₂ are the doses of drugs 1 and 2 necessary to produce IC₅₀ effect in combination. D_{x1} and D_{x2} are the doses of drugs 1 and 2 necessary to produce the same effect individually, CI<1 represents synergism, CI>1 antagonism and an average CI of 1 represents additivity [24].

3. RESULTS AND DISCUSSION

3.1. Development and Characterization of 5-FU Liposomes

The first step corresponded to the validation of a spectrophotometric assay for 5-FU quantification in liposomes. A linear relationship was found for the absorbance of the solutions between 0.25 and 6 µg/mL. The correlation coefficient obtained was 0.9991 which indicates a good linearity ($y = 0.0482x + 0.0034$, $r > 0.999$). The LOD was determined as 0.21 µg/mL and LOQ was 0.25 µg/mL. The relative standard deviation values indicated adequate accuracy and reproducibility of the assays. The method specificity was evaluated using the blank liposome and the blank liposome contaminated with a 5-FU solution of known concentration. No interference was detected and 5-FU was quantified with 95.5% and 99.4% precision and accuracy, respectively.

For 5-FU loading in liposomes with high efficiency and stability, different lipid compositions were investigated. SPC:Chol, DPPC:Chol and DSPC:Chol were combined at a

ratio 55:45 and drug to lipid ratio 0.2 based on previous studies [17]. The results of particle size/ polydispersity (PdI), zeta potential and loading efficiency are shown in Table 1.

The first series of liposomes were produced using the classical thin lipid film hydration method and the freeze/thawing cycles method. The lipid composition revealed to be critical because SPC:Chol, DPPC:Chol and DSPC:Chol resulted in 0%, 0% and $4.8 \pm 0.7\%$ loading efficiency using the classical method at 0.2 drug-to-lipid ratio (Table 1). The addition of Chol to liposomes composition increases bilayer stability and decreases the loss of hydrophilic materials, such as 5-FU [25, 26]. A small increase in 5-FU loading was observed for the freeze/thawing cycles method, as expected since this method is reported to improve the loading of hydrophilic drugs [27]. The use of repeated cycles of freezing and thawing produces the disruption of the liposomal bilayers due to the formation of ice crystals that break the lamellae of the vesicles and consequently increase the amount of hydrophilic drug-to-lipid ratio, resulting in higher drug loading [28].

The active loading using the Cu:PEI method proposed by THOMAS *et al.* [17] and the reverse phase evaporation methods [19] were also tested, both considered methods to increase hydrophilic drug loading in liposomes. It was observed for the active loading method at the 0.2 drug-to-lipid ratio a drug entrapment of approximately 17%, which means a 3.5-fold increase in loading efficiency compared to the thin lipid film hydration method. However, the use of Cu:PEI can be irritant for topical application. Additionally, the reverse phase evaporation method increased 5-FU loading to 31.5% and 43.4% when the drug to lipid ratio used was 0.2 and 0.1, respectively, which demonstrated that the drug to lipid ratio was crucial for a higher loading and is in accordance with previous observations [29]. However, low stability was found for the liposomes prepared by the reverse phase evaporation after 24 h, and aggregation occurred. Finally, using the drug to lipid ratio at 0.1 with the classical method of thin lipid hydration, the 5-FU loading efficiency obtained was near 50%. Therefore, due to the easy production, absence of residual organic solvents, longer stability and high loading efficiency, the liposomes for further studies were produced using DSPC:Chol by the thin lipid hydration method at a 0.1 drug to lipid ratio. In addition, all the liposomes prepared were smaller than 165 nm/0.3 PdI, which indicates their potential for skin delivery of drugs [30].

Regarding the zeta potential, all liposomes displayed negative/neutral zeta potential, except those obtained by active loading with Cu:PEI, due to the positive charge of PEI. The developed liposomes were stable during the 30 days of evaluation, which can be observed in Fig. (2). There was no statistically significant changes in loading efficiency and particle size ($P > 0.05$, Kruskal- Wallis test and Dunn's post-test).

Considering the necessity of an anchor for cetuximab covalent attachment to the surface of liposomes, the lipid DSPE-PEG-NH₂ was added to the formulation in order to investigate the influence of DSPE and PEGylation on physicochemical parameters and, more importantly, on 5-FU loading efficiency. Therefore, Table 2 presents the results for particle size, PdI, zeta potential and loading efficiency for 5-FU loaded liposomes, both PEGylated or non-PEGylated.

The addition of a PEG anchor on liposomes surface did not affect the particle size, zeta potential and loading efficiency of 5-FU but the PDI increased approximately 0.1, which can be related to the addition of PEG, a component with high molecular weight.

3.2. Development and Characterization of Immunoliposomes

In previous reports [31], a Chol-based anchor was used to attach cetuximab to the lipid surface of liposomes. Despite the lower cost of a Chol anchor compared to a DSPE-PEG anchor, the synthesis step can be a hurdle for large scale production. Thus, DSPE-PEG-Mal is the most frequently lipid employed for this function and the use of a linker such as DSPE prevents the lower binding of the antibody caused by the steric hindrance of PEG [32]. Therefore, the DSPE-PEG-Mal immunoliposomes were prepared and characterized, whose chromatogram is represented in Fig. (3A and 3B) for blank immunoliposomes and 5-FU immunoliposomes, respectively. The majority of cetuximab is present in fractions 4–6. Free 5-FU was released in fractions 10–14 as monitored by UV absorption (265 nm). The conjugation protocol did not alter cetuximab integrity, as demonstrated by SDS-Page electrophoresis in Fig. (3C). Under reducing conditions, 2 bands at 50 kDa and 25 kDa can be visualized, corresponding to the heavy and light chains of the antibody, respectively, which is in accordance to previous studies using IgG immunoliposomes [22].

Liposomes had an increase of approximately 30 nm in particle size, from 140 nm before functionalization to approximately 170 nm, likely due to the additional layer of cetuximab on the surface. The zeta potential also changed from -1 mV to -9 mV. Loading efficiency of 5-FU was not statistically different between non-conjugated liposomes, PEGylated liposomes and conjugated liposomes ($P > 0.05$, Kruskal- Wallis test and Dunn's post-test). Additionally, cetuximab was conjugated to the surface of liposomes (targeting efficiency) at 59.4% and 94.5% for blank liposomes and 5-FU liposomes, respectively.

3.3. Cytotoxicity Evaluation in A431 Cell Line

A431 cells, which are EGFR positive [33, 34] and responsive to cetuximab, were used to investigate the cytotoxicity induced by 5-FU and cetuximab in solution, cetuximab-conjugated and non-conjugated 5-FU liposomes. Aiming to reach the highest cytotoxicity, the treatments were performed for 72 h and 120 h, according to protocols by LU *et al.* [35]. The results of cell viability are shown in Fig. (4) and the IC₅₀ are represented in Table 3.

In all situations (Fig. 4A–D) the longer incubation time was responsible for lower cell viabilities, which indicates a time-dependent cytotoxicity. 5-FU resulted in IC₅₀ of 5.8 μ M at 72 h, similar to previous studies [36], and 8-fold reduction with longer incubation time (Table 3). Cetuximab treatment at 72 h was not able to reach IC₅₀ values; however, using 120 h incubation, the IC₅₀ obtained was 7.2 nM (Table 3). Blank liposomes were not cytotoxic in both incubation times, with cell viability above 87% for all concentrations tested (Fig. 4C, D). Highly hydrophilic drugs are known to have low intracellular absorption and may have their uptake improved by the use of liposomes [37]. 5-FU liposomes induced IC₅₀ 5-fold lower than the free drug after 72 h incubation, suggesting a key role of liposomes for 5-FU uptake and cytotoxicity. Cetuximab conjugated to liposomes was more effective after 120 h incubation and significant decrease in cell viability and IC₅₀ of 67.1

nM and 24.6 nM were obtained for 72 h and 120 h incubation, respectively. These results demonstrate that cetuximab was active after conjugation and had improved uptake and cytotoxicity for 72 h incubation compared to the solution, because cetuximab solution was not able to reach IC50 using this incubation time (Table 3).

A synergistic effect was observed for 5-FU and cetuximab both in solution (CI=0.24) or in immunoliposomes (CI=0.87), thus a major decrease in cetuximab IC50 was observed for 5-FU-loaded immunoliposomes compared to cetuximab conjugated liposomes (Table 3). Furthermore, comparing cetuximab IC50 in solution and in 5-FU immunoliposomes, after 72 h, the immunoliposomes reached IC50 and a 4-fold decrease in cetuximab IC50 was observed for 5-FU immunoliposomes after 120 h compared to cetuximab solution (Table 3).

CONCLUSION

In this study, liposomes containing 5-FU were prepared using different methods and lipid compositions. The liposomes composed of DSPC:Chol (55:45) prepared by the thin lipid hydration method and drug-to-lipid ratio 0.1 presented high 5-FU loading and stability for 30 days. Particle size remained smaller than 180 nm, making them suitable for topical application. Furthermore, successful conjugation was reached with cetuximab; the size of the liposome did not increase significantly; 5-FU loading was maintained and the antibody remained active as demonstrated in cell culture studies. In A431 cells, cetuximab had a potent cytotoxic effect after 120 h incubation and synergism was observed for the combination of cetuximab and 5-FU, in immunoliposomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank grants 2012/23764-3, # 2013/15134-2, #2014/22451-7 from Sao Paulo Research Foundation (FAPESP) and CNPQ for grant #480962/2013-8.

Biography

Raquel Petrilli

REFERENCES

1. Salehi Z, Mashayekhi F, Shahosseini F. Significance of eIF4E expression in skin squamous cell carcinoma. *Cell Biol. Int.* 2007; 31:1400–1404. [PubMed: 17689990]

2. Love WE, Bernhard JD, Bordeaux JS. Topical imiquimod or fluorouracil therapy for basal and squamous cell carcinoma: a systematic review. *Arch. Dermatol.* 2009; 145(12):1431–1438. [PubMed: 20026854]
3. Barrera MV, Herrera E. Topical chemotherapy of actinic keratosis and nonmelanoma skin cancer: current options and future perspectives. *Actas DermoSifiliogr.* 2007; 98(8):556–562. [PubMed: 17919432]
4. McGillis ST, Fein H. Topical Treatment Strategies for Non-Melanoma Skin Cancer and Precursor Lesions. *Semin Cutan Med Surg.* 2004; 23:174–183. [PubMed: 15584683]
5. Morse LG, Kendrick C, Hooper D, Ward H, Parry E. Treatment of squamous cell carcinoma with intralesional 5-Fluorouracil. *Dermatol. Surg.* 2003; 29:1150–1153. [PubMed: 14641346]
6. Naguib YW, Kumar A, Cui Z. The effect of microneedles on the skin permeability and antitumor activity of topical 5-fluorouracil. *Acta Pharm. Sin.* 2014; 4(1):94–99.
7. Rahman AM, Yusuf SW, Ewer MS. Anthracycline-induced cardiotoxicity and the cardiac-sparing effect of liposomal formulation. *Int. J. Nanomed.* 2007; 2(4):567–583.
8. Schäfer-Korting M, Korting HC, PoncePöschl E. Liposomal tretinoin for uncomplicated acne vulgaris. *Clin Investig.* 1994; 72(12):1086–1091.
9. Fresta M, Villari A, Puglisi G, Cavallaro G. 5-Fluorouracil: various kinds of loaded liposomes: encapsulation efficiency, storage stability and fusogenic properties. *Int. J. Pharm.* 1993; 99:145–156.
10. Da Costa CAM, Moraes AM. Encapsulation of 5-fluorouracil in liposomes for topical administration. *Acta Sci. Technol.* 2003; 25(1):53–61.
11. Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I, Cutsem EV. Cetuximab Monotherapy and Cetuximab plus Irinotecan in Irinotecan-Refractory Metastatic Colorectal Cancer. *N. Engl. J. Med.* 2004; 351:337–345. [PubMed: 15269313]
12. Ratushny V, Astsaturov I, Burtneess BA, Golemis EA, Silverman JS. Targeting EGFR resistance networks in head and neck cancer. *Cell. Signal.* 2009; 21:1255–1268. [PubMed: 19258037]
13. Roovers RC, Laeremans T, Huang L, De Taeye S, Verkleij AJ, Revets H, De Haard HJ, En Henegouwen PMPVB. Efficient inhibition of EGFR signalling and of tumour growth by antagonistic anti-EGFR Nanobodies. *Cancer. Immunol. Immunother.* 2007; 56:303–317. [PubMed: 16738850]
14. Cohen MH, Chen H, Shord S, Fuchs C, He K, Zhao H, Sickafuse S, Keegan P, Pazdur R. Approval Summary: Cetuximab in Combination With Cisplatin or Carboplatin and 5-Fluorouracil for the First-Line Treatment of Patients With Recurrent Locoregional or Metastatic Squamous Cell Head and Neck Cancer. *Oncologist.* 2013; 18:460–466. [PubMed: 23576486]
15. ElBayoumi TA, Torchilin VP. Tumor-Specific anti-nucleosome antibody improves therapeutic efficacy of doxorubicin-loaded long-circulating liposomes against primary and metastatic tumor in mice. *Mol. Pharm.* 2009; 6(1):246–254. [PubMed: 19049322]
16. Chakilam AR, Pabba S, Mongayt D, Iakoubov LZ, Torchilin VP. A single monoclonal antinuclear autoantibody with nucleosome-restricted specificity inhibits growth of diverse human tumors in nude mice. *Cancer Ther.* 2004; 2:353–364.
17. Thomas AM, Kapanen AI, Hare JJ, Ramsay E, Edwards K, Karlsson G, Bally MB. Development of a liposomal nanoparticle formulation of 5-Fluorouracil for parenteral administration: Formulation design, pharmacokinetics and efficacy. *J. Cont. Release.* 2011; 150:212–219.
18. Bangham AD, Standish MM, Watkins JC. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* 1965; 13:238–252. [PubMed: 5859039]
19. Elorza B, Elorza MA, Frutos G, Chantres JR. Characterization of 5-fluorouracil loaded liposomes prepared by reverse-phase evaporation or freezing-thawing extrusion methods: study of drug release. *Biochim. Biophys. Acta.* 1993; 1153:135–142. [PubMed: 8274483]
20. Uhl RG, Stevenson A, Sidorov V. Towards improved gene delivery: Flip of cationic lipids in highly polarized liposomes. *Chem. Commun.* 2007:383–385.
21. Laginha K, Mumbengegwi D, Allen T. Liposomes targeted *via* two different antibodies: Assay, B-cell binding and cytotoxicity. *Biochim. Biophys. Acta.* 2005; 1711:25–32. [PubMed: 15904660]

22. Yang T, Choi MK, Cui FD, Kim JS, Chung SJ, Shim CK, Kim DD. Preparation and evaluation of paclitaxel-loaded PEGylated immunoliposome. *J. Cont. Release.* 2007; 120:169–177.
23. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* 1984; 22:27–55. [PubMed: 6382953]
24. Jaafari A, Tilaoui M, Mouse HA, M'Bark LA, Aboufatima R, Chait A, Lepoivre M, Zyad A. Comparative study of the antitumor effect of natural monoterpenes: Relationship to cell cycle analysis. *Rev. Bras. Farmacogn.* 2012; 22:534–540.
25. Manojlovic V, Winkler K, Bunjes V, Neub A, Schubert R, Bugarski B, Leneweit G. Membrane interactions of ternary phospholipid/cholesterol bilayers and encapsulation efficiencies of a RIP II protein. *Colloids Surf. B: Biointerfaces.* 2008; 64:284–296. [PubMed: 18359207]
26. Glavas-Dodov M, Fredro-Kumbaradzi E, Goracinova K, Simonoska M, Calis S, Trajkovic-Jolevska S, Hincal AA. The effects of lyophilization on the stability of liposomes containing 5-FU. *Int. J. Pharm.* 2005; 291:79–86. [PubMed: 15707734]
27. Mayer L, Hope M, Cullis P, Janoff A. Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. *Biochim. Biophys. Acta.* 1985; 817:193–196. [PubMed: 4005257]
28. Castile JD, Taylor KMG. Factors affecting the size distribution of liposomes produced by freeze–thaw extrusion. *Int. J. Pharm.* 1999; 188:87–95. [PubMed: 10528086]
29. Schneider T, Sachse A, Robling G, Brandl M. Generation of contrast-carrying liposomes of defined size with a new continuous high pressure extrusion method. *Int. J. Pharm.* 1995; 117:1–12.
30. Verma DD, Verma S, Blume G, Fahr A. Particle size of liposomes influences dermal delivery of substances into skin. *Int. J. Pharm.* 2003; 258:141–151. [PubMed: 12753761]
31. Pan X, Wu G, Yang W, Barth RF, Tjarks W, Lee RJ. Synthesis of cetuximab-immunoliposomes *via* cholesterol-based membrane anchor for targeted delivery of a neutron capture therapy (NCT) agent to glioma cells. *Bioconjug. Chem.* 2007; 18:101–108. [PubMed: 17226962]
32. Nobs L, Buchegger F, Gurny R, Allemann E. Current Methods for Attaching Targeting Ligands to Liposomes and Nanoparticles. *J. Pharm. Sci.* 2004; 93:1980–1992. [PubMed: 15236448]
33. Su X, Yang N, Wittrup KD, Ivry DJ. Synergistic Antitumor Activity from Two-Stage Delivery of Targeted Toxins and Endosome-Disrupting Nanoparticles. *Biomacromolecules.* 2013; 14:1093–1102. [PubMed: 23444913]
34. Park SY, Yoon SJ, Freire-de-Lima L, Kin JH, Hakomori SI. Control of cell motility by interaction of gangliosides, tetraspanins, and epidermal growth factor receptor in A431 versus KB epidermoid tumor cells. *Carbohydr. Res.* 2009; 344:1479–1486. [PubMed: 19559406]
35. Lu Y, Li X, Lu H, Fan Z. 1, 9-Pyrazoloanthrones Downregulate HIF-1 α and Sensitize Cancer Cells to Cetuximab-Mediated Anti-EGFR Therapy. *PLoS ONE.* 2010; 5:e15823.
36. Lee SJ, Kim SY, Chung JH, Oh SJ, Ryu JS, Hong YS, Kim TW, Moon DH. Induction of thymidine kinase 1 after 5-fluorouracil as a mechanism for 30-deoxy-30-[18F]fluorothymidine flare. *Biochem. Pharmacol.* 2010; 80:1528–1536. [PubMed: 20723540]
37. Eloy JO, De Souza MC, Petrilli R, Barcellos JPA, Lee RJ, Marchetti JM. Liposomes as carriers of hydrophilic small molecule drugs: Strategies to enhance encapsulation and delivery. *Colloids Surf. B.* 2014; 123:345–363.

Fig. (1).
Schematic representation of active drug loading and reverse phase evaporation methods.

Fig. (2). 5-FU loaded liposomal stability at 4°C for 30 days: particle size and loading efficiency of DSPC:Chol (55:45) liposomes at 0.1 drug-to-lipid ratio prepared by the thin lipid hydration method.

Fig. (3).

A) Chromatogram for blank immunoliposomes and **B)** 5-FU immunoliposomes and **C)** SDS-Page electrophoresis profile of free cetuximab and cetuximab-conjugated blank and 5-FU immunoliposomes, respectively.

Fig. (4).

Cell viability studies for A431: **A)** 5-FU solution; **B)** cetuximab solution; **C)** blank liposomes, 5-FU liposomes and immunoliposomes after 72 h treatment and **D)** blank liposomes, 5-FU liposomes and immunoliposomes after 120 h treatment.

Table 1

Effect of composition, drug/ lipid ratio and preparation methods on 5-FU loading efficiency in liposomes.

Composition	Technique	Drug/lipid ratio	Particle size (nm)	PdI	Zeta Potential (mV)	Loading efficiency
SPC:Chol	Thin film hydration	0.2	133.5 ± 4.2	0.306 ± 0.03	-10.01 ± 0.40	0%
	Freeze thawing cycles	0.2	129.1 ± 5.4	0.212 ± 0.09	-6.57 ± 0.15	0%
DPPC:Chol	Thin film hydration	0.2	117.8 ± 2.7	0.374 ± 0.04	-10.25 ± 0.77	0%
	Freeze thawing cycles	0.2	103.9 ± 2.5	0.280 ± 0.06	-9.78 ± 1.01	4.9 ± 0.4%
DSPC:Chol	Thin film hydration	0.2	143.2 ± 3.8	0.207 ± 0.02	-13.60 ± 1.41	4.8 ± 0.7%
	Thin film hydration	0.1	143.4 ± 11.4	0.276 ± 0.08	-1.07 ± 0.47	49.9 ± 2.1%
	Freeze thawing cycles	0.2	162.4 ± 3.2	0.231 ± 0.08	-7.77 ± 1.03	7.7 ± 0.5%
	Active loading Cu:PEI	0.2	148.9 ± 1.0	0.260 ± 0.03	12.25 ± 0.35	17.2 ± 5.8%
	Reverse phase evaporation	0.2	139.4 ± 3.4	0.201 ± 0.08	*	31.5 ± 0.2%
	Reverse phase evaporation	0.1	127.2 ± 2.2	0.206 ± 0.05	*	43.4 ± 5.9%

The results represent the mean ±SD of 3 determinations.

* Aggregation occurred after 24 hs and zeta potential could not be assessed.

Table 2

Comparison of non-PEGylated and PEGylated liposomes for particle size, PdI, zeta potential and loading efficiency.

Liposome	Size (nm)	PdI	Zeta potential (mV)	Loading efficiency (%)
Blank	142.3±8.6	0.252±0.09	-4.44±0.18	-
5-FU	143.4±11.4	0.276±0.08	-1.07±0.47	49.9±2.1
PEGylated blank	147.7±9.3	0.351±0.18	0.47±1.09	-
5-FU PEGylated	131.4±7.8	0.361±0.16	0.47±0.38	44.5±5.0

Table 3

IC50 values for different formulations after 72 h or 120 h incubation in A431 cells.

Sample	5-FU IC50 (μ M) as a function of incubation time		Cetuximab IC50 (nM) as a function of incubation time	
	72 (h)	120 (h)	72 (h)	120 (h)
5-FU solution	5.8	0.7	-	-
Cetuximab solution	-	-	*	7.2
5-FU liposomes	1.1	0.6	-	-
Cetuximab-conjugated blank liposomes	-	-	67.1	24.6
Cetuximab-conjugated 5-FU liposomes	2.2	0.6	8.5	1.8

* IC50 was not reached.