Antitumor Effect of 5-Fluorouracil-Loaded Liposomes Containing n-3 Polyunsaturated Fatty Acids in Two Different Colorectal Cancer Cell Lines

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Abstract. It has been shown that long-chain n-3 polyunsaturated fatty acids (n-3 PUFAs) could act synergistically with 5-fluorouracil (5-FU) to kill cancer cells. To facilitate their simultaneous transport in the bloodstream, we synthesized, for the first time, liposomes (LIPUFU) containing 5-FU in the aqueous core and docosahexaenoic acid (DHA)/eicosapentaenoic acid (EPA) at a ratio of 1:2 in the lipid bilayer. LIPUFU were stable with a uniform size of 154 ± 4 nm, PDI of 0.19 ± 0.03 and zeta potential of -41 ± 2 mV. They contained 557 ± 210 μmol/l DHA, 1467 ± 362 μmol/l EPA, and 9.8 ± 1.1 μmol/l 5-FU. Control liposomes without (LIP) or with only 5-FU (LIFU) or n-3 PUFAs (LIPU) were produced in a similar way. The effects of these different liposomal formulations on the cell cycle, growth, and apoptosis were evaluated in two human colorectal cancer (CRC) cell lines differing in sensitivity to 5-FU, using fluorescence-activated cell sorting analyses. LIPUFU were more cytotoxic than LIP, LIFU, and LIPU in both LS174T (p53+/+, bax−) and HT-29 (p53−/−, bax+) cell lines. Similar to LIFU, LIPUFU increased the percentage of cells in S phase, apoptosis, and/or necrosis. The cytotoxic potential of LIPUFU was confirmed in vivo by tumor growth inhibition in the chicken choioallantoic membrane model. These results suggest that LIPUFU could be considered to facilitate the simultaneous transport of 5-FU and n-3 PUFAs to the tumor site, in particular in case of CRC liver metastases.

KEY WORDS: colorectal cancer; chemotherapy; 5-fluorouracil; polyunsaturated fatty acids; liposomes.

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the second leading cause of cancer death (1, 2). Moreover, CRC incidence and mortality are constantly increasing worldwide because of the aging population and the adoption of harmful Western diet and sedentary lifestyle (1). Therefore, CRC prevention, screening, and treatment are among the main public health concerns. If CRC is diagnosed in the early stages by colonoscopy or sigmoidoscopy, complete cure can be obtained by surgical resection of the tumor with sufficient margins (3). However, when the disease has reached stage III/IV and spread to the lymph nodes or distant organs, adjuvant chemotherapy is required to prevent local recurrence and metastatic invasion (2). The reference drug in CRC treatment is 5-fluorouracil (5-FU), an antimetabolite that causes cell cycle arrest in S phase after conversion into fluoronucleotides and misincorporation into RNA and DNA (4). Usually, 5-FU is co-administered with folinic acid, oxaliplatin, and/or irinotecan as FOLFIRI, FOLFOX, or FOLFOXIRI regimen (5). However, 5-FU is unstable, with a short biological half-life of 13 ± 7 min (6), and targets indifferently dividing cancer and normal cells, thus causing serious adverse effects, such as diarrhea (7.1–13.6%),
nausea/vomiting (23.0%), leucopenia (2.9–12.5%), anemia (6.2%), and mucositis (14.3%) (7). Therefore, new strategies have been proposed to enhance the therapeutic index of 5-FU. Among them, 5-FU administration in nanosized carriers, such as nanoparticles, micelles, or liposomes, has been already carried out successfully (8). In particular, several studies have evaluated 5-FU entrapment in liposomal formulations with targeting ligands, such as folic acid, to overcome the drawbacks associated with passive targeting (9–13). In another approach, 5-FU co-administration with natural and safe compounds that exhibit anticancer properties, such as long-chain n-3 polyunsaturated fatty acids (n-3 PUFAs), has been proposed to reduce not only the effective therapeutic dose of 5-FU but also non-specific toxicity and cachexia associated with systemic chemotherapy (14, 15). Several studies have indeed shown that supplementation with n-3 PUFAs had a powerful adjuvant activity in combination with 5-FU (16–18). To our knowledge, however, there is no study having attempted to combine these two approaches in order to protect them from rapid degradation in the bloodstream and facilitate their simultaneous transport to the tumor site, which may be particularly relevant in the bloodstream and facilitate their simultaneous transport to the tumor site, which may be particularly relevant in the context of liver metastases (19). Therefore, the aim of this study was to encapsulate 5-FU in the aqueous core of liposomes containing n-3 PUFAs in their lipid bilayer and to evaluate in vitro and in ovo their therapeutic efficacy in different CRC models.

MATERIALS AND METHODS

Liposome Preparation

The classic thin film hydration method of Bangham et al. (20) was optimized to produce a liposomal formulation (LIPUFU) containing n-3 PUFAs in the lipid bilayer and 5-FU in the aqueous core. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (both from Chemie Brunschwig AG, Basel, Switzerland) were added at a molar ratio of 1:2 to a phospholipid mixture of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, 1,2-dipalmitoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (DPPG), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) (all from Corden Pharma, Liestal, Switzerland). The compounds were dissolved in 15 ml chloroform for 3 h on vortex at room temperature. A film was obtained after complete solvent evaporation under vacuum at 474 mbar for 1 h and 30 min at 43°C. The film was rehydrated in a 5-FU solution (kindly provided by the pharmacy of the Geneva University Hospital) adjusted with phosphate-buffered saline (PBS: BioConcept Ltd., Allschwil, Switzerland) at pH 7.4. Multilamellar vesicles were obtained by vortexing at room temperature for 15 min and moderate shaking on a rotavapor at 53–55°C for 30 min. The lipid suspension was then left at room temperature in the dark for 24 h. Single unilamellar vesicles of homogenous size were obtained by sonication for 5 min at a 20% amplitude (Branson Ultrasonics, Danbury, USA). After sonication, the liposomal suspension was transferred into a 15-ml Falcon tube and stored at 4°C. To remove unentrapped 5-FU, EPA, and DHA, the liposomal suspension was ultracentrifuged at 40,000 rpm for 3 h and washed with 2 ml of water three times successively. Sonication and ultracentrifugation steps were used after early experiments showed that extrusion and dialysis steps gave low incorporation rates of 5-FU and n-3 PUFAs (data not shown). The final liposomal suspension was stored in 1× PBS at pH 7.4 after filtration through a 0.22-μm Millipore membrane.

Control liposomes without (LIP) or with only 5-FU (LIFU) or n-3 PUFAs (LIPU) were produced in a similar way.

Liposome Characterization

Diameter, polydispersity index (PDI), and zeta potential of the different liposomal formulations were measured by photon correlation spectroscopy at 90° angle and room temperature, using a Zetasizer Nano ZS (Malvern Instruments, Ltd., UK). Liposomal suspension uniformity and morphology were further evaluated with transmission electron microscopy (Tecnai G2 20 TEM, FEI Company, OR, USA), Deposit grids (formvar carbon film, 200 mesh copper grids, Electron Microscopy Sciences, Hatfield, USA) were prepared by ionization, and 5-μl samples of the different liposomal suspensions were dropped off on them for 30 s. The grids were then washed twice in a drop of water for 2 s, dried, then left for 30 s in a drop of saturated uranyl acetate, and finally dried. Three pictures were taken at different places and magnifications for each sample.

Loading Efficiency of 5-FU

Loading efficiency and release of 5-FU were evaluated by high-performance liquid chromatography (HPLC). 5-FU dilutions were performed to obtain a standard curve of 0.1, 0.5, 1, 1.3, 10, 100, 250, 500, and 1000 μmol/l. Liposomal suspension samples of 100 μl were lyzed in 200 μl PBS with 2% Triton X-100 (Sigma-Aldrich, Buchs, Switzerland) and 5 μl 5-bromo-2’-deoxyuridine (BrUrd; Sigma-Aldrich, Buchs, Switzerland) as internal control. Ten-microliter samples were injected into a HPLC system equipped with a UV detector and data analysis software (HPLC W600 controller, W2487dual, 717 plus autosampler, Waters Corporation, MA, USA). Peak separation was performed on a C-18 reverse phase column of 3.9 × 10.5 μm (Atlantis Waters, MA, USA) at a flow rate of 1.0 ml/min and UV detection of 266 nm. The mobile phase (pH = 4.47, 25°C) consisted of 136 g NaCH₃COO, 77 g NH₄CH₃COO in 1 l H₂O, and 250 ml CH₃COOH 10%. The retention time was 4.5 min for 5-FU and 8.8 min for BrUrd. The standard curve for 5-FU was linear from 0.013 to 130 μg/ml (r² = 0.99). 5-FU loading efficiency was calculated according to the calibration curve, by dividing the amount measured into the liposomal suspension with the initial amount used in the liposomal formulation.

Fatty Acid Composition of the Liposomal Membranes

Fatty acid composition of the liposomal membranes was analyzed by capillary gas chromatography (GC). Total lipids were extracted from 300-μl liposomal suspension in 6 ml chloroform-methanol 2:1 (v:v) containing 0.1% butylated hydroxytoluene. Diphosphatidyl marginal acid was added as internal standard. The extract was washed with 1.5 ml NaCl...
and the mixture was vortexed 10 min at 2000 rpm and centrifuged 10 min at 4000 rpm. The organic phase was
transferred into glass tubes with screw caps and evaporated for 35 min at 45°C and 275 mbar, using the RapidVap®
system (VWR International LLC, PA, USA). The lipid extract was resuspended in 80 µl dichloromethane, and 25 µl
Methyl-Prep II (Grace Davison, Bannockburn, IL) was added to the mixture before incubation for 10 min at room
temperature to allow fatty acid conversion into the corresponding fatty acid methyl esters (FAMEs). FAMEs
were then extracted by evaporation for 5 min at 40°C and 250 mbar, using the RapidVap® system, and resuspended in
300 µl iso-octane/butylated hydroxytoluene. After vortexing for 10 s, the suspension was centrifuged for 2 min at 5000 rpm
and the supernatant was transferred into autosampler vials.
FAME composition was determined by injecting 1-µl samples
through the split injector (ratio 25) at 60°C into a GC device
operating at detector temperatures of 250°C. A Select FAME
column of 50 m × 0.25 mm ID × 0.25-mm film thickness was
used for FAME separation (Agilent Technologies, Santa
Clara, USA). Hydrogen was used as the carrier gas at a
flow rate of 2.8 ml/min, with nitrogen as the make-up gas for the
flame ionization detector. The temperature ramp was
programmed to rise from 60 to 250°C at a rate of 12°C/min
and then kept constant for 7 min at 250°C to wash the
column. FAME identification was obtained by comparison
with the retention times of pure standard mixtures. The retention time was 15.38 and 16.71 min for EPA and DHA,
respectively. The fatty acid amount (µmol/l) was quantified by
integrating the peak and adjusting the results with the
internal standard. Incorporation efficiency of DHA and/or
EPA was calculated by dividing the amount actually incorpo-
rated into the liposomal suspension with the initial amount
used in the liposomal formulation.

Cell Lines and Culture

Two human colorectal adenocarcinoma cell lines, LS174T (ATCC no. CL-188™, USA) and HT-29 (ATCC no.
HTB-38™, USA), were chosen according to their different
genetic background and sensitivity to 5-FU. According to
Violette et al. (21), LS174T (p53 +/+, bax−/−) shows greater
resistance to 5-FU than HT-29 (p53−/0, bax+/+). They were
kept in exponential growth phase at 37°C and 5% CO 2 by
subculturing twice a week in Dulbecco’s modified Eagle’s
medium supplemented with 10% heat-inactivated fetal bo-
ine serum and 0.1 g/l penicillin-streptomycin (all from
Invitrogen, Zug, Switzerland).

Table I. Description of the Different Liposomal Formulations

| Type   | Nb | Size (nm) | PDI  | Zeta (mV) | 5-FU (µmol/l) | DHA (µmol/l) | EPA (µmol/l) | Ratio   |
|--------|----|-----------|------|-----------|---------------|--------------|--------------|---------|
|        |     | Mean ± SD | Mean ± SD | Mean ± SD | Initial | Final | (%) | Initial | Final | (%) | Initial | Final | (%) | D H A / EPA |
| LIP    | 3   | 146 ± 5   | 0.15 ± 0.02 | −41 ± 4 | - | - | - | - | - | - | - | - | - |
| LIFU   | 3   | 155 ± 9   | 0.18 ± 0.05 | −40 ± 5 | 1000 | 7.1 ± 1.8 | (0.7) | - | - | - | - | - | - |
| LIPU   | 3   | 150 ± 3   | 0.16 ± 0.02 | −43 ± 3 | - | 1522 | 601 ± 156 | (39.5) | 3306 | 953 ± 458 | (28.8) | 0.63 | - |
| LIPUFU | 4   | 154 ± 4   | 0.19 ± 0.03 | −41 ± 2 | 1000 | 9.8 ± 1.1 | (1.0) | 1522 | 557 ± 210 | (36.6) | 3306 | 1467 ± 362 | (44.4) | 0.38 |

SD standard deviation, PDI polydispersity index, mV millivolts, 5-FU 5-fluorouracil, DHA docosahexaenoic acid, EPA eicosapentaenoic acid, LIP liposome without 5-fluorouracil and n-3 polyunsaturated fatty acids, LIFU liposome with 5-fluorouracil, LIPU liposome with n-3 polyunsaturated fatty acids, LIPUFU liposomes with 5-fluorouracil and n-3 polyunsaturated fatty acids, Nb number.
Cytotoxicity Assays

The cytotoxic potential of the different liposomal formulations was evaluated on the two cell lines, using fluorescence-activated cell sorting (FACS) analyses. Cells were seeded 24 h before treatment at a density of 20,000 cells/well in 24-well plates (BD Biosciences, Allschwil, Switzerland). After treatment, cells were detached with 0.2 ml trypsin-EDTA 1× (Life Technologies, Zug, Switzerland), washed with 1 ml PBS 1×, and centrifuged for 10 min at 1200 rpm. The pellet was then resuspended in 2 μg/0.4 ml propidium iodide (PI: BD Biosciences, Allschwil, Switzerland) to allow discrimination between permeable cells labeled with PI (dead) and unlabeled cells (living). The number of PI-labeled and unlabeled cells per microliter was then counted using a BD Accuri C6 flow cytometer (BD Biosciences, Allschwil, Switzerland) at excitation and emission wavelengths of 488 and 530 nm, respectively. FACS analysis was carried out with the corresponding Accuri C6 software.

Cell Cycle Modulation

One of the main mechanisms of action of 5-FU is to block the cell cycle in S phase by inhibition of the thymidylate synthase. Therefore, the cytostatic effect of the liposomal emulsions was also evaluated by adding to the previous protocol a denaturation step before PI staining of the nuclei. After detachment and centrifugation, cells were fixed by adding, drop by drop while vortexing, 0.4 ml of cold 70% ethanol into the cell pellet and then stored > 2 h at −20°C. At the day of analysis, cells were washed twice to remove the ethanol and resuspended in PI/RNase staining buffer (BD Biosciences, Allschwil, Switzerland) at a concentration of 10^6 cells/0.5 ml. Cells were then incubated 15 min in the dark at room temperature and stored at 4°C before FACS analysis as previously described.

Apoptosis Induction

Apoptosis induction by the different liposomal suspensions was quantified using a two-parameter FACS analysis with annexin V/PI detection kit according to the manufacturer’s instruction (BD Biosciences, Allschwil, Switzerland). Briefly, cells were detached with trypsin-EDTA 1×, washed with PBS 1×, and then resuspended in binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl_2) at a concentration of 1 × 10^6 cells/ml. Samples were stained with 5 μl annexin V conjugated with fluorescein isothiocyanate (FITC) and 5 μl PI at room temperature for 15 min in the dark. They were then diluted in 0.4-ml binding buffer and analyzed within 1 h using a BD Accuri C6 flow cytometer at excitation and emission wavelengths of 488 and 530 nm, respectively.
Chorioallantoic Membrane Assay

A first in vivo evaluation of the antitumor potential of the liposomes was performed on human CRC tumors xenografted on chorioallantoic membranes (CAMs) of chick embryos lacking B and T cell-mediated immunity (22). Fertilized chick eggs were placed on rotating grids in an incubator (37°C, 60% humidity), with the narrow apex down for 3 days. At embryo development day 3 (EDD3), a hole was drilled into the eggshell at the narrow apex, covered with adhesive tape to avoid dehydration, and returned into the incubator. At EDD7, the hole was enlarged to 1–2 cm. With a needle, CAM was scratched close to a blood vessel or around a junction of several blood vessels, and a silicon O-ring (Apple Rubber Products Inc. Lancaster, USA) was deposited at this place. Before implantation in the O-ring, cells were treated with 0.5% trypsin-EDTA 1×. The last resuspension was done in a nutriment solution containing 50% Matrigel (BD Biosciences, Belford, USA)/50% medium in order to obtain a concentration of 5 × 10⁶ cells in 20 μl. To avoid desiccation and contamination, the window on the eggshell was sealed with parafilm and the eggs were returned into the incubator until the day of treatment. At EDD 12, either a sham treatment with PBS or the LIPUFU suspension was injected i.v. into the main blood vessel through a 33″-gauge needle at a volume of 25 μl. Tumor growth was assessed at 24 h, 48 h, and 72 h after PBS or LIPUFU injection by means of image recording and bi-dimensional measurements of the tumor size using a binocular microscope (Leica M205FA microscope, objective × 10, FOV: 1.5052 mm²).

\[
\text{Tumour surface} = \pi \left[ 3(a + b) - \sqrt{(3a + b)(a + 3b)} \right]
\]
Statistical Analysis

Every experiment was performed in quadruplicate samples. The variables were expressed as proportions or means ± 1 standard deviation (SD), as appropriate. Differences between the different treatment conditions were analyzed with one-way ANOVA followed by a post hoc Tukey’s multiple comparisons test after checking the normal distribution and equality of variance of the data with the skewness and kurtosis test and the Bartlett’s test, respectively. In case of unequal variance, the Kruskal-Wallis test was used, followed by the two-sample t test for comparison between two treatment conditions. All statistical analyses were performed with the Stata/IC 13.1 software for Windows (StataCorp LP, College Station, TX, USA). Statistical significance is reported as follows: *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \).

RESULTS

Liposomal Formulation

Different preparation procedures were evaluated to obtain stable liposomes of uniform size with an optimal concentration of n-3 PUFAs and 5-FU. The most favorable LIPUFU formulation was obtained by adding 2.5 mg DHA and 5 mg EPA at a ratio of 1:2 to a phospholipid mixture of 78 mg DPPC, 64 mg cholesterol, 4.2 mg DPPG, and 2.1 mg DSPE-PEG2000. The rehydration solution consisted of 0.65 mg 5-FU in 5 ml PBS at pH = 4. This formulation gave liposomes with uniform size of 154 nm, PDI of 0.19, and zeta potential of \(-42 \text{ mV} \) (Fig. 1 and Table I). As evaluated by GC analysis, the incorporation rate of DHA and EPA into the lipid bilayer was 37% and 44%, corresponding to 557 \( \mu \text{mol/l} \) DHA and 1467 \( \mu \text{mol/l} \) EPA. HPLC analysis showed that the entrapment rate of 5-FU in the aqueous core of the liposomes was only 1% corresponding to 9.8 \( \mu \text{mol/l} \) 5-FU (Table I).

LIPUFU Cytotoxicity

The cytotoxic potential of LIPUFU was compared with that of LIP, LIFU, and LIPU in the two HT-29 and LS174T cell lines. While LIP had no effect compared to untreated controls, LIPUFU, and to a lesser extent LIFU and LIPU, significantly decreased the growth (Fig. 2a) and viability (Fig. 2b) of both cell lines. As expected, HT-29 cells were more
sensitive to LIFU containing 5-FU than LS174T cells, while LS174T cells appeared to be more sensitive to LIPU containing n-3 PUFAs than HT-29 cells. LIPUFU was more cytotoxic than LIP and LIFU in both cell lines \((P < 0.01)\) and LIPU in HT-29 cells \((P < 0.001)\) (Fig. 2).

**Cell Cycle Modulation**

Since 5-FU blocks the cell cycle in the S phase, the cytostatic potential of LIP, LIFU, LIP, and LIPUFU was evaluated in LS174T (Fig. 3a) and HT-29 (Fig. 3b) cells. As expected, LIFU and LIPUFU acted similarly in both cell lines by significantly decreasing the percentage of cells in the G1 phase and increasing the percentage of cells in the S phase, whereas LIPU containing the n-3 PUFAs seemed to act differently by blocking LS174T cells in the G1 phase, but had no effect on HT-29 cell cycle.

**Apoptosis Induction**

An increase in the percentage of apoptotic cells was only observed in HT-29 cells treated with LIFU \((4.8 \pm 0.7\%, P = 0.002)\) or LIPUFU \((3.3 \pm 0.5\%, P = 0.001)\) compared to untreated controls \((1.8 \pm 0.2\%)\). LIPUFU increased the percentage of necrotic cells in both LS174T \((10.3 \pm 3.3\% \text{ vs } 5.1 \pm 1.0\%, P = 0.02)\) and HT-29 \((14.5 \pm 0.7\% \text{ vs } 3.1 \pm 2.7\%, P = 0.004)\) cells compared to untreated controls.

**In Vivo Tumor Toxicity**

A first \textit{in vivo} assessment of the antitumor potential of LIPUFU was performed in the CAM model. Since it was not possible to obtain solid LS174T tumors in this model, only the growth of HT-29 tumors was measured over a 3-day period after a sham treatment with PBS or with LIPUFU. Overall, PBS-treated tumors grew more or less rapidly, whereas LIPUFU-treated tumors tended to not grow or even decrease in size (Fig. 4).

**DISCUSSION**

Any enhancement of the therapeutic index of 5-FU would be of great value in the management of advanced CRC patients. Among the various strategies envisaged, 5-FU entrapment in synthetic carriers has been evaluated with more or less success to facilitate its delivery to tumor site.\(^{10,\text{ }}\)
Another mechanism may involve could induce cell cycle arrest and apoptosis of HT-29 and n-3 PUFA enterments were not optimal, though 5-FU and n-3 PUFA entrapments were not optimal, successfully synthesized with a good reproducibility. Although 5-FU and n-3 PUFA entrapments were not optimal, a strong cytotoxic effect was observed on both HT29 and LS174T cell lines. These first results indicate that such an approach could be envisaged in CRC chemotherapy to reduce the effective therapeutic dose and thus toxicity of 5-FU on healthy tissues with rapid cellular turnover.

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