Pharm Res (2013) 30:1281–1290
DOI 10.1007/s11095-012-0967-2

RESEARCH PAPER

In Vitro and *In Vivo* Evaluation of Lipofufol, a New Triple Stealth Liposomal Formulation of Modulated 5-Fu: Impact on Efficacy and Toxicity

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Received: 13 September 2012 / Accepted: 14 December 2012 / Published online: 6 February 2013

ABSTRACT

Purpose Drug resistance and severe toxicities are limitations when handling 5-FU. We have developed a triple liposomal formulation of 5-FU combined to 2′-deoxyinosine and folinic acid to improve its efficacy-toxicity balance.

Methods Stealth liposomes were obtained using the thin-film method. Antiproliferative activity was tested on human colorectal and breast cancer models using sensitive (HT29) and resistant (SW620, LS174t, MDA231) cell lines. *In vivo*, pharmacokinetics, biodistribution and safety studies were performed in rodents. Finally, efficacy was evaluated using two tumor-bearing mice models (LS174 and MDA231) with response and survival as main endpoints.

Results LipoFufol is a 120-nm pegylated liposome, displaying 20–30% encapsulation rates. *In vitro*, antiproliferative activities were higher than 5-FU, and matched that of FolFox combination in colorectal models, but not in breast. Drug monitoring showed an optimized pharmacokinetics profile with reduced clearance and prolonged half-life. Liposome accumulation in tumors was shown by fluorescence-based biodistribution studies. Beside, milder neutropenia was observed when giving LipoFufol to animals with transient partial DPD-deficiency, as compared with standard 5-FU. In LS174t-bearing mice, higher response and 55% longer survival were achieved with Lipofufol, as compared with 5-FU.

Conclusion The issues of drug-resistance and drug-related toxicity can be both addressed using a stealth liposomal formulation of modulated 5-FU.

KEY WORDS 5-FU · colorectal cancer · pharmacokinetics · stealth liposomes · toxicity

INTRODUCTION

5-Fluorouracil (5-FU) is one of the most widely used antineoplasic agents for treating a variety of solid tumors (1). However, resistance (e.g., Thymidylate synthetase (TS) overexpression in tumors) may lead to limited efficacy at the bedside (2). Additionally, 10–20% of patients treated with standard 5-FU usually show severe toxicities. DPD deficiency, a pharmacogenetic syndrome leading to limited detoxification capabilities, makes patients overexposed and prone to toxicities, thus often hampering treatment completion, when not directly life-threatening (3). In this respect, improving toxicity/efficacy balance of 5-FU remains a challenging issue in oncology. Drug delivery systems are developed for both enhancing the concentration of anticancer agents in tumours while reducing their distribution and toxicity against normal tissues (4,5). Liposomes are canonical carriers that present several advantages because of the sustained release of the encapsulated drugs and their excellent biocompatibility (6). Therefore, incorporating 5-FU into liposomes could be an attractive approach for improving drug efficacy and reduction of side effects. The combination of chemotherapeutics agents with modulators is another promising strategy to overcome the limitations of conventional cancer treatment. 2′

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deoxyinosine (d-Ino), a purine nucleoside, was previously tested on the bench to modulate tumor metabolism and to boost cytotoxicity of 5-FU. In previous studies, we demonstrated that d-Ino provides tumor cells with deoxy-ribose-1-phosphate, the cofactor of thymidine phosphorylase necessary for the near-direct conversion of 5-FU to active FdUMP in cancer cells (7). d-Ino has been therefore selected as a promising agent that proved to improve the antitumour action of 5-FU, including in several resistant models (7,8). Despite significant achievements in enhancing 5-FU efficacy in various experimental models, *in vivo* handling of d-Ino was rendered difficult because of its dramatic erythrocytic catabolism, requiring to the use of extremely elevated doses (e.g., up to 3.2 g/kg) to achieve some efficacy when combined with 5-FU *in vivo*. To overcome this, we first developed a liposomal form of d-Ino (9), then a co-encapsulated formulation of 5-FU and d-Ino, with siginificant improvements in 5-FU efficacy (10). However, as the standard modulation of 5-FU in clinical setting is its combination with folinic acid (FA) because it stabilizes the ternary complex FdUMP-TS-tetrahydrofolate, it has been decided here to further develop a new triple stealth liposomal formulation of 5-FU associated now with both d-Ino and FA to be tested in *in vitro* and *in vivo* cancer models, with efficacy, pharmacokinetics and toxicity as main endpoints.

MATERIALS AND METHODS

Cell Lines

Experiments were carried out on a panel of resistant/sensitive human cancer cells: colon carcinoma cell lines (ie: HT29 (sensitive), SW620 (resistant) and LS174t (resistant)) and breast cancer cells MDA231 (resistant) stably transfected with luciferase. Cells were maintained in RPMI supplemented with 10% fetal calf serum, 10% penicillin, 1% kanamycin in a humidified CO_2 incubator at 37°C. All experiments were performed in exponentially growing cells. Colorectal Cell lines came from ATCC, USA. MDA231-Luc2 cells were purshased from Calliper LS (Villepinte, France).

Drugs and Chemical

Egg yolk phosphatidylcholine (PC), phosphatidylglycerol (PG), cholesterol (Chol) and methoxypolyethylen glycol (PEG), 2′-deoxyinosine (d-Ino), 5-FU, folinic acid (FA) and dimethyl sulphoxide were purchased from Sigma (St Quentin Fallavier, France). All lipids were of pharmaceutical grade. Di-kalium hydrogenous phosphate (K_2HPO_4) buffer, tetrabutyl ammonium nitrate, acetonitrile, ether and methanol were bought from CarboErba (Milano, Italy). Culture media and reagents was purchased from Eurobio (Courtaboeuf, France). DIR was purchased from Perkin Elmer Caliper LS (Villepinte, France). Oxaliplatin came from Teva U.S.

Liposomal Preparation

Phosphatidylcholine, phosphatidylglycérol, cholesterol and methoxypolyethylen glycol were mixed in a 52%, 5%, 41.5% and 1.5% proportion. LipoFufol was generated following the standard thin-film method: lipid mixture in methanol was evaporated under nitrogen in a round bottom flask to form a dried thin film. This thin film was then hydrated with 5-FU, folinic acid and d-Ino (molar ratio 1/1/1) dissolved in carbonate buffer (pH 7.2–7.4). The resulting loaded PEG-liposomes were then shaken. Homogenous size distribution as a SUV population was obtained by 5 min of continuous sonication (24 KHZ, amplitude 20) with a probe (UIS250L, Hielscher, Germany) of the preparation maintained refrigerated in ice. To remove the non-encapsulated drug, the liposomal suspension was next centrifuged with 30 Kd Vivaspin 0.22 μm filters (ViVaScience, Sartorius France) at 10 000 rpm during 180 min at 4°C. The liposomal suspension was re-suspended in carbonate buffer. Finally, sterile liposomes were obtained after extrusion through PVDF filters (Durapore 0.22 μm, Millipore, Molsheim, France).

Liposome Size Determination

Mean LipoFufol diameter and size distribution was determined by dynamic light scattering at constant temperature (25°C) and a 90° scattering angle after dilution in phosphate buffer. Average diameters and size distributions (polydispersity index, PDI) were evaluated, a z-average performing a single exponential fit to the correlation function (cumulants analysis) with the implemented software. Dynamic light scattering measures the motion of particles in a medium of known viscosity and refractive index. The Stokes–Einstein equation ($D=kT/6\pi R\eta$ where D is the particle scattering coefficient, T the temperature, k the Boltzmann constant, R the particle radius and η the viscosity of the solvent) links the correlation function to the hydrodynamic radius allowing to access to the hydrodynamic particle diameter. Particle diameter was determined by dynamic light scattering on a Zeta Sizer NanoSeries Malvern (Malvern Instruments, Venissieux, France). Measurements were performed in triplicate before and after purification. Ten measures were performed at each time point for the evaluation of the colloidal stability.

Encapsulation Rates and Stability

Encapsulation rate was evaluated by quantifying drugs entrapped in liposomes, as compared with initial amounts

used in the preparation. Stability was monitored up to 1 month following various storage conditions (room temperature, 4°C, frozen). Quantification of 5-FU, d-Ino and FA into liposomes was carried out by reversed-phase HPLC analysis (HP-1100, Agilent France). Separation was performed at ambient temperature on a Macherey Nagel C18 column (Macherey-Nagel, France). The mobile phase consisted of 0.1 M K_2HPO_4 and methanol. A linear gradient elution program was applied (0–40% methanol from 0 to 8.6 min, then 40% until the end of the run). Chromatographic separation was performed at 0.4 then 1 ml/min and monitored at 266 nm. Retention times were 5.1, 9.2 and 10.8 min for 5-FU, d-Ino and FA, respectively. 5-Bromo-uracil was used as internal standard (RT: 8.1 min). Data collection and analysis were performed using Chemstation software (Agilent, France).

Antiproliferative Assay

Cells were maintained in standard culture condition. All experiments were performed when cell growth was in exponential phase. Human colorectal cells HT29, SW620, LS174t or breast cancer cells MDA231 were seeded at a density of 5×10^4 cells per well in 96-well plates. After overnight attachment, exponentially growing cells were exposed to increasing concentrations (0.01 to 1000 μM) of 5-FU alone, combined with folinic acid and d-Ino, or LipoFufol (0.01 to 1000 μM eq. 5-FU) after 72 h of exposure with gentle rocking. Additional combination with oxaliplatin was further tested in SW620 and MDA231 cells only. Cell viability was evaluated using the classic colorimetric MTT test (11). The IC50 was defined as the 5-FU concentration inhibiting 50% of cell growth. In a preliminary experiment, effect of empty liposomes and lipidic mix on cell proliferation was tested.

Pharmacokinetics and Safety Studies in Rats

LipoFufol (eq. 15 mg/kg 5-FU and 20 mg/kg d-Ino) or free 5-FU (15 mg/kg) associated with free d-Ino (20 mg/kg) were administered intravenously in wistar male rats. Blood samples (200 μl) were withdrawn at T0, T15, T60, T120, T180min for PK analysis. 5-FU and d-Ino were assayed by UV-HPLC after liquid-liquid extraction as described previously, with minor modifications (10). 5-FU and d-Ino pharmacokinetics parameters were best estimated with a one-compartment model using Kinetic-Pro software. Additionally, LipoFufol (eq. 15 mg/kg 5-FU) or standard 5-FU (15 mg/kg) combined with modulators were administered intravenously in wistar male rats pretreated with 10 mg/kg uracil I.V. to induce a transient and partial DPD-deficiency. Blood samples were withdrawn on Day 0 (D0, control), D2, D6, D8, D11 and D14 for neutrophils count as a marker for drug-related toxicity. Experiments were in agreement with the animal welfare guidelines of our institution, and local animal ethics committee approval was obtained prior to starting the experiments.

Biodistribution Studies in Mice

Fifteen thousands MDA-231 cells were injected orthotopically into the mammary fat pad in female nu/nu mice ($n=5$). Fifteen days after, animals were injected intraperitoneally with 180 mg/kg luciferin potassium salt (Caliper file sciences, Roissy, France) before undergoing anesthesia with isoflurane in an anesthesia induction box. Then 2% isoflurane in O_2 was continously delivered via a nose cone system in the dark box of a high sensitivity CDD camera cooled to 90° C (IVIS Spectrum Imager, Perkin Elmer Caliper, USA). Bioluminescence acquisition for tumor localization was performed 15 min after substrate injection. Mice were treated with 30 mg/kg of Lipofufol previously tagged with a fluorescent probe (DIR, λabs 750 nm, λem 782 nm; Perkin Elmer Caliper, France). Detection of signal-tumor localization and size was performed using the Living Image 4.2 software (Perkin Elmer Caliper, USA). Biodistribution of liposomes was monitored every hour over 12 h. Additional fluorescent acquisition was performed on D2 and D7. Mouse care was in agreement with the animal welfare guidelines of our institution, and local animal ethics committee approval was obtained prior to starting the experiments.

In Vivo Efficacy Study

Experiments in tumor-bearing animals were performed using two resistant models (colorectal LS174t ectotopic and breast MDA231 orthotopic xenografts). Preliminary experiments on satelitte groups were performed to check that empty liposomes displayed no impact on tumor growth as compared with saline.

LS174t Model. Four–week old female Swiss, nude mice ($n=8$ per group, Janvier, France) were subcutaneously inoculated with 1×10^6 LS174t cells on the right flank. Ten days after implant, and once tumors had reached an accurately measurable size, mice were treated with 30 mg/kg 5-FU given either alone, combined with free d-Ino (40 mg/kg) and FA (80 mg/kg) or as LipoFufol at the same dosages. Drugs were administered intraperitoneally on a 3-times per week basis for 3 consecutive weeks. Tumor size was measured 3-times a week in three dimensions using vernier calipers, and tumors weight (mg) was calculated using the following standard formula: $\text{mass}=\pi/6\times\text{lenght}\times\text{width}\times\text{height}$ (12). Animal weight was monitored thrice a week as a marker of general toxicity.

MDA231 Model. Four-week old female Swiss, nude mice (n= 12 per group, Janvier, France) were inoculated in mamary fat pad with 1×10^5 MDA231 cells. Ten days after implant, mice were treated with 30 mg/kg 5-FU given either alone, combined with free d-Ino (40 mg/kg) and FA (80 mg/kg) or as LipoFufol (same dosages). Drugs were administered by intraperitoneal injection on a 3-times per week basis for 5 consecutive weeks. Tumor size, localization and metastasis spreading were measured by bioluminescence following the same procedure than described previously using the Ivis Spectrum imaging system and the Living Image 4.2 software (Perkin Elmer Caliper, U.S.A.). Animal weight was monitored thrice a week as a marker of general toxicity.

Mouse care was in agreement with the animal welfare guidelines of our institution, and local animal ethics committee approval was obtained prior to starting the experiments. Animals were sacrificed when showing signs of cachexia, impaired mobility, signs for distress, or when tumor reached an apparent weight of 3 g.

Statistical Analysis

When sample size was sufficient, data were analyzed using R statistical software (http://www.R-project.org). All results are expressed as means ± standard errors (SE). Multiple mean comparisons were performed using the Kruskal-Wallis non-parametric test at α=0.05 when necessary.

RESULTS

Liposome Preparation

Mean diameter of liposomal suspension was 120 nm (92%) and 240 nm (8%). In this trial the percentage of encapsulation for the three drugs varied from 23 to 26%. LipoFufol proved to be stable for at least 1 month at room temperature 25°C without alteration of either liposomal diameter or drug encapsulation rate. When stored at 4°C, liposomal suspension was altered and size distribution was not satisfactory anymore. Similarly, stability of frozen liposomes was not satisfactory (data not shown).

In Vitro Efficacy Study

In SW620 cells, antitumour effect of liposomal 5-FU was found to be 14.5-fold higher than that of the free drug (Fig. 1). IC50 values were 2±0.5 μM (LipoFufol: liposomal 5-FU + d-Ino + FA combination), 29±4 μM (5-FU), 14±2 μM (free: 5-FU + d-Ino + FA combination), and 1.6±1 μM (5-FU + FA + oxaliplatin). Similar increase in efficacy was further confirmed in HT29 and LS174t cells exposed to liposomal 5-FU as compared with standard 5-FU (Fig. 2a and b). In MDA231 cells, IC50 values were 5.7±1 μM (LipoFufol: liposomal 5-FU + d-Ino + FA combination), 6.1±2 μM (5-FU), 6±1.8 μM (free: 5-FU + d-Ino + FA combination), and 7±1 μM (5-FU + FA + oxaliplatin).

Pharmacokinetics and Toxicity Studies in Rats

Pharmacokinetics study in rodents showed that pharmacokinetics profile of liposomal 5-FU is modified as compared with that of standard 5-FU (Fig. 3). In normal rats, LipoFufol leads to 62% slow-down in 5-FU total clearance (0.231 l/h *vs.* 0.087 l/h). Early sampling times (i.e., up to 60 min) showed no statistical differences in 5-FU levels after standard 5-FU and LipoFufol administration. However, a significant difference was observed in late (i.e. 120 and 180 min) sampling times ($p<0.05$). Transient DPD deficiency led to marked differences in 5-FU levels in rats treated with the free form (AUC = 28.5 VS. 2.2 mg/ml.h, i.e. x12.9-fold increase) whereas smaller difference was observed between animals administered with liposomal 5-FU (AUC= 7.56 VS. 2.96 mg/ml.h, i.e. x2.55-fold increase). When considering rats with transient partial DPD-deficiency, treatment with free 5-FU led to a 3.76 higher plasma exposure as compared with liposomal 5-FU (AUC= 28.5 *VS.* 7.56 mg/ml.h^{-1}). Conversely, monitoring of d-Ino levels showed little difference between rats with DPD-deficiency and normal rats without DPD impairement. However, a marked difference was observed in d-Ino levels administered as a free drug as compared with the liposomal form. Due to chromatographic interferences and because of the weak signals measured, it was not possible to measure accurately d-Ino levels after 30 min when administered as free drug and therefore PK parameters were not calculated (Fig. 4). We observed a milder (78 *VS.* 100%) and shorter (3 *VS.* 8 days) neutropenia in rats pre-treated with 5-FU exposed to LipoFufol as compared with free 5-FU. Final recovery from neutropenia was observed at 10 and 12.5 days for both treatments (Fig. 5).

Biodistribution Studies in Mice

Figure 6 displays the typical changes in signals measured in animals injected with fluorescent liposomes. A slow distribution throughout the body was observed. Transient liver uptake was observed 4–5 h after administration, and LipoFufol accumulation in tumor surroundings was better observed up to 7 days after administration.

In Vivo Efficacy in Mice

Empty liposomes showed no antiproliferative activity (data not shown). In LS174t-bearing mice, tumor size was monitored

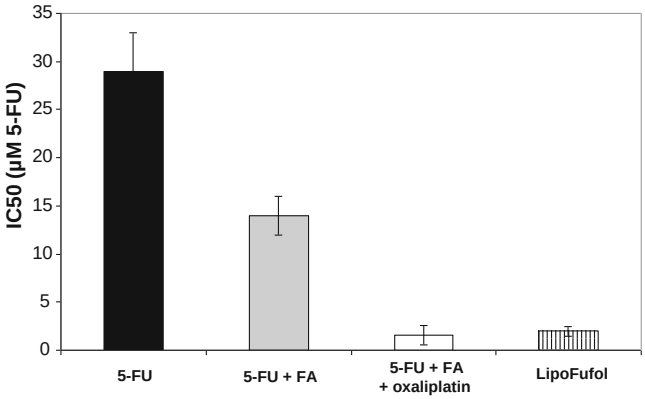

Fig. 1 Modulation of 5-FU cytotoxicity on SW620 cells. Cells were exposed to 5-FU alone, in combination with 1 mM d-Ino + FA, 1 μM oxaliplatin + FA or to LipoFufol. Cell viability was measured spectrophotometrically after MTT staining after 72 h of continuous exposure.

and compared until all groups were measurable (ie, D24). Mean tumor weight at study conclusion were 1922 ± 580, 1733 ± 520, 2255 ± 797 and 984 ± 264 mg in control, 5-FU, modulated 5-FU, and liposomal 5-FU respectively (Fig. 7).

This difference was found to be statistically different and the tumor size in animals treated with liposomal 5-FU was significantly lower as compared with the other groups ($p < 0.05$, Anova with Kruskal Wallis non parametric testing). Median survival (Fig. 8) was 22 days (control), 20 days (5-FU), 17 days (free combination) and up to 31 days (Liposomal 5-FU, i.e. + 51% increase in life span as compared with control group, +55% as compared with standard 5-FU and +82% as compared with the free association).

In MDA231-bearing mice, mean tumor size as evaluated by bioluminescence at study conclusion were $4.8.10^8 \pm 2.5$ p/s, $4.9.10^8 \pm 2.7$ p/s, $6.10^8 \pm 3.9$ p/s and $6.4.10^8 \pm 3.6$ p/s in control, 5-FU, modulated 5-FU, and liposomal 5-FU respectively (Fig. 9). No difference in tumor size was evidenced at study conclusion ($p > 0.05$, Anova with Kruskal-Wallis non parametric testing). Median survival was 28 days in control group and in modulated 5-FU respectively, and 34 days in mice treated with standard 5-FU and Lipofufol. For both models, no signs of toxicity were observed in animals, regardless of the treatment modalities, and no statistical differences were found in animal weights among these different groups (data not shown).

DISCUSSION

Considering the lack of specificity of most anticancer agents and their relatively limited efficacy at the bedside, encapsulating anticancer drugs to improve their clinical benefit remains an attractive strategy (13,14). Standard chemotherapies are limited by their inability to deliver specifically drugs to the tumors and by their toxicity on healthy tissues. Severe treatment-related adverse events lead to delay in the administration of the forthcoming courses. In this respect, improving the toxicity/efficacy balance of 5-FU remains a challenging issue in oncology, because this drug is widely used for treating a large variety of tumors. Over the last decade, different strategies for optimizing

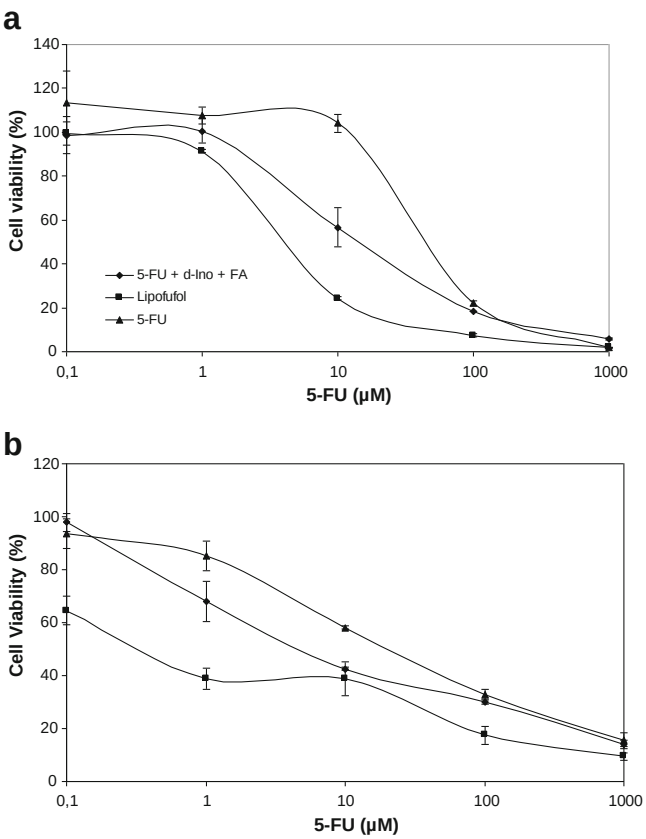

Fig. 2 Modulation of 5-FU cytotoxicity on LS174t (**a**) and HT29 (**b**) colorectal cells. Cells were exposed for 72 h to 5-FU alone, as a free combination, or as the liposomal combination. Cell viability was measured spectrophotometrically after MTT staining.

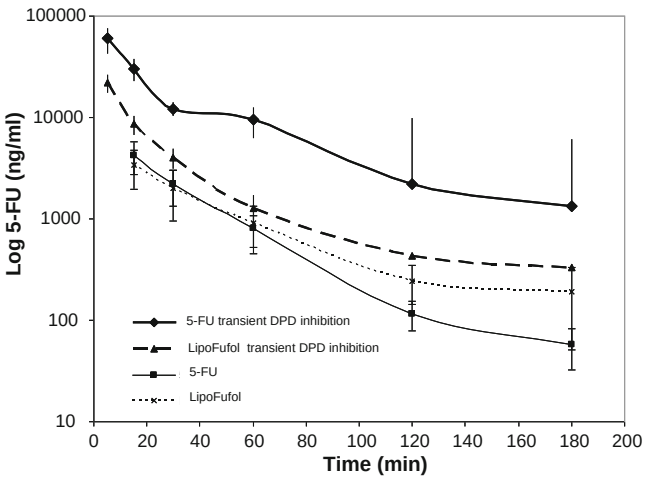

Fig. 3 Pharmacokinetics profile of 5-FU in rats obtained after iv injection of 15 mg/kg 5-FU as a free form or as a liposomal formulation. Experiments were performed in normal individuals and in rats pre-treated with 10 mg/kg uracil to mimic a partial transient DPD-deficiency.

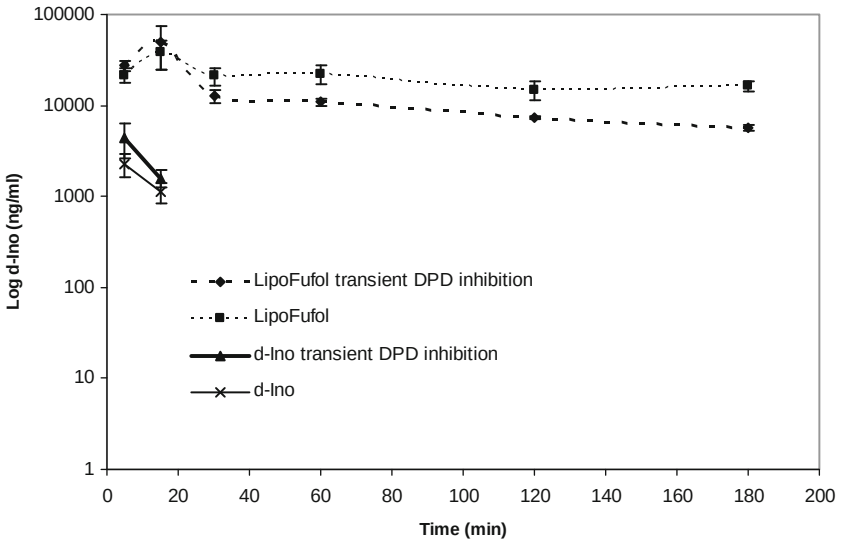

Fig. 4 Pharmacokinetics profile of d-Ino in rats obtained after iv injection of 20 mg/kg d-Ino as a free form or as a liposomal formulation. Experiments were performed in normal individuals and in rats pre-treated with 10 mg/kg uracil to mimic a partial transient DPD-deficiency. No d-Ino was quantifiable accurately anymore after 20 minutes when administered as free form.

the use of fluoropyrimidines have emerged from the better understanding the cellular and clinical pharmacology of these agents. Suicide gene-therapy (15), chronopharmacology-guided schedules (16,17), pharmacokinetically-guided dosing (18) and modulating strategies (19,20) have been tested as a means to improve 5-FU efficacy, with various successes. Additionally, developing innovative druggability approaches proved to be a relevant strategy (21–23). Small liposomes can accumulate in tumors through the enhanced permeability and retention (EPR) effect, as a result of tumour blood vessel leakiness. This, combined with lower sequestration rates in healthy organs (24–26), make that higher efficacy and minimal side-effects are expected when developing drug-loaded liposomes. Our group has previously developed a first liposomal formulation encapsulating 5-FU + d-Ino, a modulator designed to boost intratumoral formation of anti-TS FdUMP metabolite (10). Because combining FA to 5-FU is a standard in clinical oncology (a.k.a. Fufol or LV-5-FU protocols), we have tried here to further develop an original triple combination [5-FU + d-Ino + FA] encapsulated in a single carrier. Multiple encapsulation in a single liposome is often considered as a challenge because usually, it means dealing with active compounds displaying different physico-chemical properties and solubility profiles, thus making the determination of optimal encapuslation conditions particurlarly tricky. Our manufacturing process was optimized and proved to be rapid, simple and able to generate reproducible batches in term of particle size and encapsulation rates. Mean encapsulation rates were about 25-30%, a value acceptable regarding the hydrophilic characteristics of the three compounds we mixed. In our previous study encapsulation rates were 10% and 26% for 5-FU and d-Ino respectively. This improvement in encapsulation rates was achieved by optimizing the separation step between encapsulated from non-encapsulated drugs (e.g., reduction from 16 H at 70 000 g to 180 min at 12 000 g centrifugation), thus preventing the obtained liposomes to be degraded during the manufacturing process. At the bedside, a major drawback of 5-FU is its poor pharmacokinetics profile due to rapid elimination with a half-life of about 8–20 min, and a high inter-patient variability because of erratic DPD activities in patients mostly related to circadian variations and genetic polymorphism (27). DPD activity has been shown to convert about 85–90% of administered 5-FU to inactive metabolite FUH2, and patients with impaired DPD are likely to experience severe/lethal

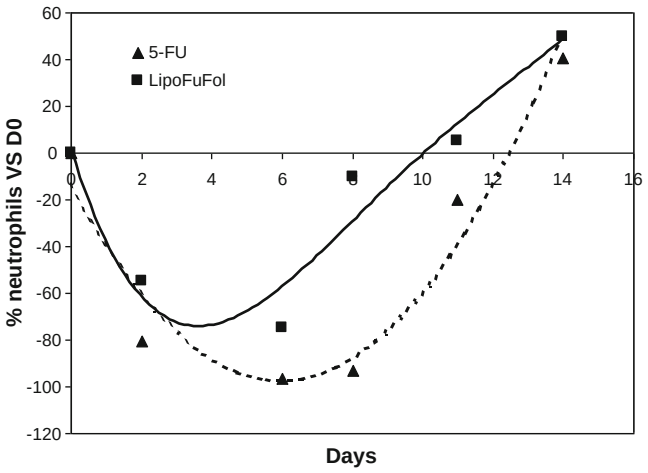

Fig. 5 Toxicity studies: shorter and milder neutropenia was observed in partial transient DPD-deficient rats exposed to Lipofufol (15 mg/kg) as compared with animals treated with standard 15 mg/kg of standard 5-FU. Partial transient DPD deficiency was achieved by pre-treating the animals with 10 mg/kg uracil.

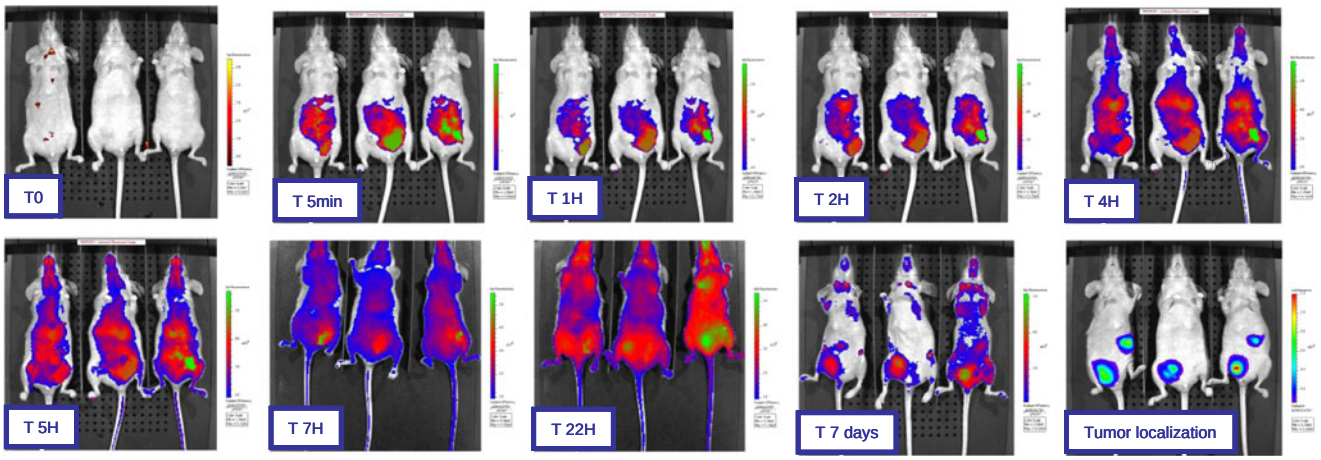

Fig. 6 Biodistribution study of LipoFuFol in tumor-bearing mice. Liposomes were tagged with fluorescent DIR prior to I.P. injection (30 mg/kg). Tumor localization was achieved by luciferine injection and bioluminescence acquisition.

toxicities when given standard dosage (28). We hypothesized that bypassing liver uptake with stealth liposomes would partly skip the DPD-deficiency issue. Pharmacokinetics and toxicity studies were performed in rats, rather than mice, because several blood samples could be withdrawn throughout time from a same animal for both PK and blood count monitoring, thus reducing the total number of animals to be used and complying with current animal welfare guidelines. Transient partial DPD-deficiency was achieved by pre-treating animals with high-dose uracil, as in the UFT formulation (29). We observed that animals pre-treated with uracil and treated with LipoFufol exhibited milder and shorter neutropenia as compared with standard 5-FU. Here, neutrophils count was considered indeed as a surrogate for 5-FU-induced hematological toxicities, because severe neutropenia has been frequently described in patients with DPD-deficiency after 5-FU intake. Overall, LipoFufol proved to be safely administered, including in individuals with impaired detoxification ability. Interestingly, differences in 5-FU levels between normal individuals and those with transient partial DPD-deficiency were lower when using LipoFuFol than when using free 5-FU, thus suggesting that changes in liver metabolism could be less problematic when using a liposomal formulation of 5-FU, because stealth liposomes have been proved to partly bypass liver uptake (30). Developing a liposomal formulation of 5-FU such as LipofuFol would not probably be sufficient to ensure a complete safety in patients with total DPD deficiency. However, one can assume that the issue of partial DPD deficiency, observed in 10–15% of patients (31) could be addressed by using a stealth liposomal drug with a reduced liver uptake. Additionally, pharmacokinetic studies showed a decrease in 5-FU Kel when using LipoFufol as compared with free 5-FU, with an increase in plasma half-life from 15 to 35 min (ie, +133%), thus suggesting that infusion duration could be reduced as compared with standard 5-FU, which usually requires several days of continuous infusion in clinical practice (32). Similarly, a marked change in d-Ino PK profile was observed when using the liposomal formulation because the liposomes protect d-Ino from extensive erythrocytic

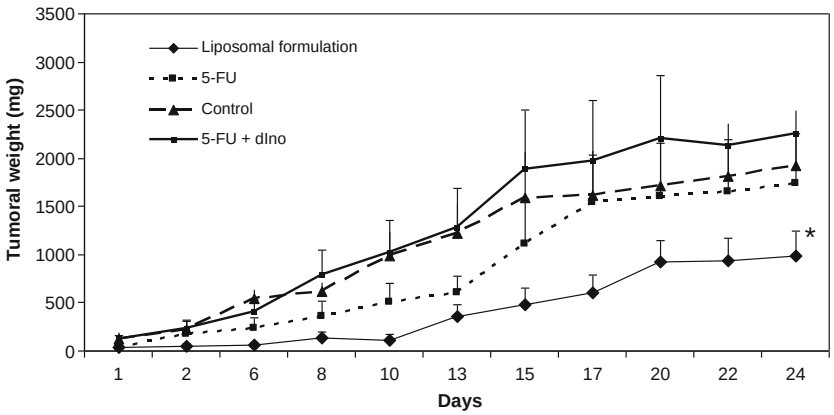

Fig. 7 Effects of the liposomal association on LS174t tumour growth in nude mice. Animals ($n = 8$ per group) were subcutaneously transplanted. Treatment were administered I.P. for three consecutive days, over 3 consecutive weeks with each of the following : saline, 5-FU 30 mg/kg, 5-FU 30 mg/kg combined with d-Ino or liposomal 5-FU at 30 mg/kg. A statistical difference was observed between the groups at study conclusion (Anova with Kruskal-Wallis testing, $p < 0.05$).

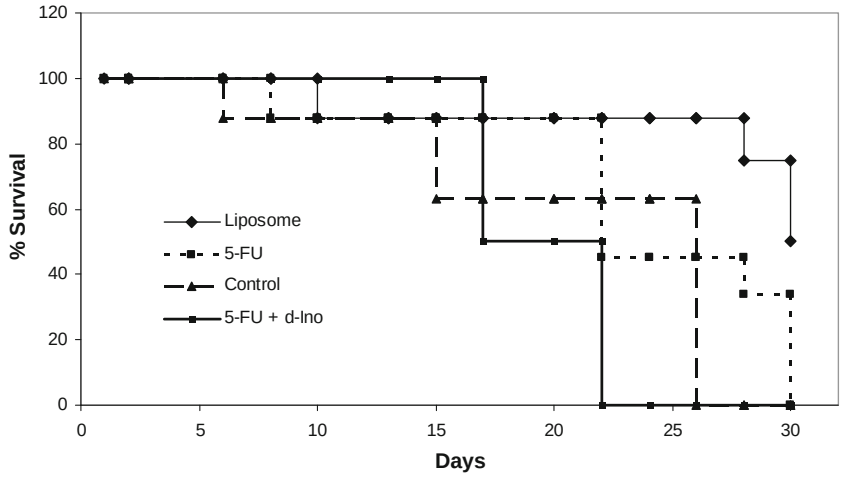

Fig. 8 Kaplan-Meyer representation of mice survival in tumor-bearing animals treated with saline, 5-FU 30 mg/kg, 5-FU 30 mg/kg combined with free drugs or liposomal 5-FU 30 mg/kg. A 51% increase in survival was achieved in mice treated with liposomes, whereas standard 5-FU failed in improving survival duration as compared with controls and the free association seemed to be deleterious.

catabolism, an observation in line with previous *ex-vivo* experiments (9). Of note, pre-treating animals with uracil did not impact markedly on d-Ino levels, an observation consistent with the metabolic pattern of this drug. The higher tumor selectivity of Lipofufol was next evaluated by biodistribution studies in tumor-bearing mice. 5-FU is normally rapidly catabolized by hepatic DPD, and 90–95% of the drug is admittedly cleared in the liver minutes after the administration (28). Here, we observed a mild and transient liver uptake at 5 h, followed by maximal accumulation in tumours 7 days post injection, thus suggesting that better selectivity could be achieved indeed using liposomal 5-FU. Because this formulation was designed to overcome drug resistance through better TS inhibition in colorectal cancer models, we have tested lipoFufol efficacy *in vitro* on a limited but representative panel of sensitive (HT29) and resistant (LS174t, SW620,) cell lines. Additionally, Lipofufol was further tested in a 5-FU-resistant human breast cancer cell line (MDA231). *In vitro*, liposomal 5-FU proved to increase 5-FU sensitivity by 14-time in SW620, 14-time HT29 and 10-time in LS174t but not in MDA231 breast cancer cells. Of note, we found that *in vitro* LipoFufol efficacy matched that of a Folfox-like association, one of the standard combinational regimen for treating metastatic colorectal cancer. Finally, we have tested Lipofufol efficacy *in vivo* using two different models. The LS174t model that proved *in vitro* to be sensitive was tested to confirm *in vivo* the gain in sensitivity previously observed. Additionally, and despite lack of evidence for improved sensitivity *in vitro*, we decided to further test the MDA231 breast

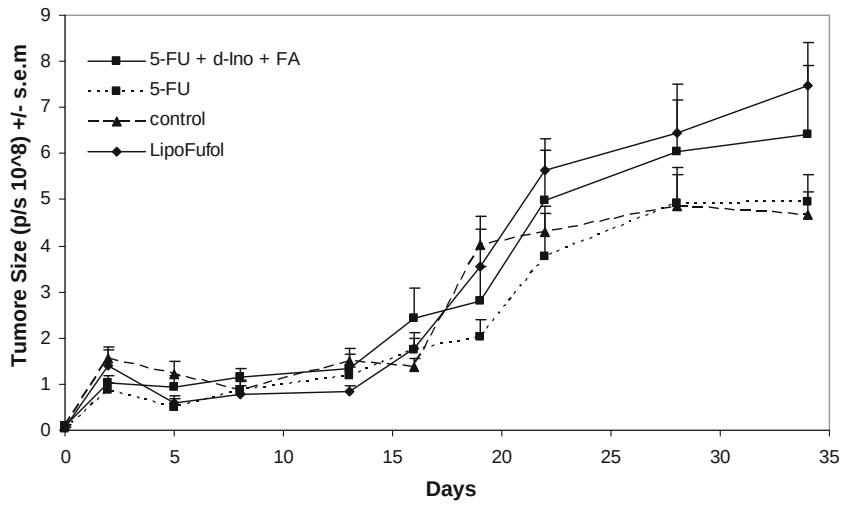

Fig. 9 Efficacy study in MDA231 bearing mice. Animals ($n = 12$ per group) were orthotopically transplanted on the mammary fat pad with Luc + −MDA231 tumoral cells. Treatment were administered I.P. for three consecutive days, over five consecutive weeks with each of the following : saline, 5-FU 30 mg/kg, 5-FU 30 mg/kg combined with d-Ino + FA or LipoFufol 30 mg/kg. No statistical difference was observed between the groups (Anova with Kruskal-Wallis testing, $p > 0.05$).

cancer model *in vivo*, because liposomal formulation are always supposed to exhibit a better efficacy in *in vivo* settings as compared with *in vitro* experiments. Most liposomal formulations are designed indeed to optimize the PK profile of drugs, an improvement that can not been properly evaluated *in vitro*.

In LS174t-bearing mice, lipoFufol proved to significantly reduce tumour growth by 43% and to stretch survival times by 55% as compared with standard 5-FU. Of note, standard 5-FU combined with free d-Ino failed in improving treatement efficacy, much probably because free d-Ino is rapidly cleared by extensive erythrocytic catabolism when it is not protected in a liposome (9). Furthermore, and despite the absence of signs for toxicity, both 5-FU and the free association seemed to have deleterious effects, because median survivals were shorter as compared with the control group. In MDA231-bearing mice, as for *in vitro* experiments, no gain in efficacy was evidenced, although a non-significant trend towards longer survival was observed in mice treated with LipoFufol. These *in vivo* data confirm therefore that the MDA231 breast cancer cells do not respond to the liposomal formulation. We can only speculate about the possible reasons for this lack of efficacy. Unfavourable TP/DPD ratio in MDA231 cells resulting in limited tumoral activation and extensive detoxification towards FUH2 could at least partly explain this (33). Still, understanding why MDA231 fails in responding to LipoFufol remains challenging. Specific tumor mico-environnement and interstitial pressure in the mammary fat pad model could explain the lack of efficacy, in addition to the above-mentionned unfavourable molecular profile. In this respect, designing smaller liposomes could overcome this and achieve some better efficacy in MDA-231 bearing mice.

CONCLUSION

We have developed an original liposomal formulation of modulated 5-FU designed to exhibit strong antiproliferative activity, even towards drug-resistant colorectal models, while being less toxic than 5-FU. Animal pharmacokinetics show that sustained and higher drug exposure can be achieved upon administration of LipoFufol as compared with standard 5-FU. However, when administrated to rats with transient partial DPD-deficiency, LipoFufol proved to be less toxic than standard 5-FU, much probably due to a limited liver uptake that makes DPD activity less crucial for its disposition. LipoFufol proved to improve the pharmacokinetic profile of 5-FU, prolonging its circulation time due to the pegylated materials which results in tumor accumulation as confirmed by biodistribution study in tumor-bearing mice. Although ineffective on resistant breast cancer MDA231 cells, LipoFufol proved to restore *in vitro* the sensitivity of highly resistant human colorectal cell lines such as SW620 and LS174t models. Of note, this increase in efficacy was further confirmed in LS174t xenografted mice with a +55% survival time. Overall, LipoFufol proved to be a safer and powerful alternative to standard 5-FU in experimental treatment of colorectal cancer, both *in vitro* and *in vivo*.

ACKNOWLEDGMENTS

This study was supported by generous grants from the GEFLUC Marseille-Provence and the Association pour la Recherche sur le Cancer (ARC, Grant #1026).

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