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RESEARCH PAPER

Biodistribution, Tumor Uptake and Efficacy of 5-FU-Loaded Liposomes: Why Size Matters

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ABSTRACT

Purpose We have investigated the impact of particle size on the biodistribution, tumor uptake and antiproliferative efficacy of 5-FU-loaded liposomes.

Methods Three different batches of pegylated liposomes varying in size (i.e., 70, 120 and 250 nm respectively) were tested. The active compounds encapsulated were an equimolar mix of 5-FU, 2′-deoxyinosine and folinic acid. Liposomes were subsequently tested on the human breast cancer model MDA231 cells, a model previously found to be resistant to 5-FU. *In vitro*, antiproliferative efficacy and microscopy studies of liposomes uptake were carried out. *In vivo*, comparative biodistribution and efficacy studies were performed in tumor-bearing mice.

Results Difference in size did not change *in vitro* antiproliferative activity. Fluorescence-Microscopy studies showed that liposomes were mainly uptaken by tumor cells through a direct internalization process, regardless of their size. Biodistribution profiles in tumor-bearing mice revealed higher accumulation of small liposomes in tumors throughout time as compared with normal and large liposomes ($p < 0.05$). Additionally, we observed that the bigger were the tumors, the more vascularised they were and the greater was the difference in accumulation between small and large liposomes. Consequently, *in vivo* efficacy studies showed at study conclusion that a 68% reduction in tumor size was achieved with small liposomes ($p < 0.05$), whereas larger liposomes failed to reduce significantly tumor growth. Similarly, at study conclusion a trend towards higher survival-rate in animals treated with smaller liposomes was observed.

Conclusion This study suggests that particle size is critical to achieve higher selectivity and efficacy in experimental oncology, including in resistant tumors.

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INTRODUCTION

Treatment of solid tumors could be improved by delivering greater dose of active compounds to the tumor while sparing healthy tissues. With conventional drugs, cytotoxic effects on normal tissues (e.g, bone marrow, heart, digestive tract) are usually dose-limiting, thus impeding the efficacy of the chemotherapy eventually because of a poor efficacy/toxicity balance (1,2).

Innovative drug delivery systems aim at increasing the concentration of a therapeutic agent in the tumor while limiting systemic exposure, thus improving the selectivity of the treatment (3–5). A large variety of technologies have been developed to reach this goal, such as liposomes, micelles, or nanoparticles (6,7). Most of these approaches are based upon the observation that leaky tumor vasculature will help nanoparticles to accumulate in tumor surroundings, thus improving their selectivity. This property known as the EPR (Enhanced Permeability Retention) Effect (8), depends mainly on the particle size. Intuitively, smaller liposomes are expected to better accumulate in tumor surroundings than bigger ones, because the cut-off of the pores is believed to be in the 100–150 nm range (9,10).

We have previously developed a 100–120 nm stealth liposomal formulation of 5-FU associated with two modulators, 2′ deoxyinosine (d-Ino) and folinic acid (FA). 2′deoxyinosine is expected to trigger the intratumoral activation of 5-FU towards the 5-fluoro-deoxyuridine monophosphate (FdUMP) active metabolite (11,12), whereas folinic acid stabilizes next the ternary complex FdUMP-TS-5,10-methylene tetrahydrofolate, thus maximizing the thymineless-related apoptosis induction. This liposome proved to be highly effective against a variety of colorectal models, including resistant ones (e.g., LS174t,

SW620 cells), but failed to exhibit similar efficacy against the resistant MDA 231 human breast cancer cells (13). Understanding why breast cancer cells fail in responding to liposomal 5-FU is challenging. Specific tumor mico-environment and interstitial pressure in the mammary fat pad model we used could explain the lack of efficacy, in addition to unfavourable molecular profile (14). In this respect, designing smaller liposomes could lead to greater tumor uptake through optimized EPR effect, thus possibly achieving some better efficacy in initially refractory MDA-231-bearing mice. The objective of the present work was therefore to compare the biodistribution, tumor uptake and antiproliferative efficacy of three different batches of pegylated liposomal of 5-FU varying in size (i.e., 70, 120 and 250 nm) so as to study to what extent particle size could be a determinant of antitumor efficacy in the highly resistant MDA-231 breast cancer model (15).

MATERIALS AND METHODS

Cell Lines

Experiments were carried out on 5-FU-resistant human breast cancer cells MDA231 stably transfected with luciferase (MDA231-LUC2). Cells were maintained in RPMI supplemented with 10% fetal calf serum, 10% penicillin, 1% kanamycin in a humidified CO2 incubator at 37°C. All experiments were performed in exponentially growing cells. MDA231-Luc2 cells were purchased 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 sulfoxide were purchased from Sigma (St Quentin France). DIR a fluorophore tag was purchased from Caliper France. Di-kalium hydrogenous phosphate (K_2HPO_4) buffer, tetrabutyl ammonium nitrate, acetonitrile, ether and methanol were from CarloErba (Milano, Italy). Culture media was purchased from Eurobio (France). DIR was purchased from Caliper LS (Villepinte France). AngioSense 750 fluorescent imaging agent was from Perkin-Elmer USA.

Preparation of Stealth Liposomes

Phosphatidylcholine (PC), phosphatidylglycerol (PG), cholesterol (Chol) and methoxypolyethylen glycol (PEG) were mixed in a 52%, 5%, 41.5% and 1.5% proportion. Liposomal preparation was further mixed with DIR as a fluorophor tag when required. All components were of pharmaceutical grade. The three batches varying in mean diameter (lipo-50, lipo-100 and lipo-250) were obtained following the standard thin-film method. This film was then hydrated with an isotonic carbonate solution (pH 7.2) containing an equimolar mix of 5-FU, 2′-deoxyinosine and folinic acid for 30 min at 40°C. Multilamellar vesicles were formed by vortex mixing the lipid dispersions at room temperature. Homogeneous size distribution as SUV was achieved by sonication using the probe UIS250L (Hielscher, Germany) during 1 min, 5 min (frequency: 100) and 10 min (frequency: 50) to generate three different particle sizes of < 70 (i.e., Lipo-70), 120 (i.e., Lipo-120) and 250 nm (i.e., Lipo-250), respectively. Removal of the non-encapsulated drug was achieved by centrifugation at 10 000 rpm for 2 h30 at 4°C using Vivaspin filter (30 000 kD). Resulting liposomes were re-suspended in sterile carbonate buffer before use.

Liposome Size Determination

Mean LipoFufol diameter and size distribution was determined by dynamic light scattering at constant temperature (25°C) with a 90° scattering angle after dilution in phosphate buffer. Both average diameters and size distributions (polydispersity index, PDI) were evaluated 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.

Encapsulation Rates and Stability

Encapsulation rate was evaluated by quantifying drugs entrapped in liposomes, as compared with the initial amounts used when starting the preparation. Quantification of 5-FU and d-Ino into liposomes were carried out by UV-HPLC analysis (HP-1100, Agilent France), as described previously. Chromatography 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). All analytes were monitored at 266 nm. Data collection and analysis were performed using Chemstation software (Agilent, France).

In Vitro Antiproliferative Assay

Human breast cancer cells MDA231 were seeded at a density of 6×10^4 cells per well in 96-well plates. After overnight attachment, exponentially growing cells were exposed to

increasing concentrations (0.01 to 1,000 μM) of 5-FU alone, combined with folinic acid and d-Ino, or as a liposomal formulation (lipo-70, lipo-120 and lipo-250) over 72 h with gentle rocking. Cell viability was evaluated using the classic colorimetric MTT test (16). The IC50 was defined as the 5-FU concentration inhibiting 50% of cell growth. All experiments were performed on two separate plates, and further replicated in a least one independent experiment.

In Vitro Immunofluorescence Staining

Cells were grown on Labtek chamber slides (Nunc, Roskilde, Denmark) and were incubated with the liposomes for 1, 2 and 4 h. Then, cells were fixed in ice-cold methanol and incubated with for 2 min with 0.25 μg/mL 4′,6-diamidino-2-phenylindole (DAPI ; Sigma) in dark, before being harvested in PBS. After mounting in antifading plate (Invitrogen), cells were observed using a Leica DM-IRBE microscope coupled with a digital camera driven by Metamorph® software (Princeton Instrument, USA).

Biodistribution Studies in Mice

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. Fifteen thousand MDA-231 cells in 60% matrigel were injected orthotopically into the mammary fat pad of female nu/nu mice ($n=5$ per condition). Tumor localization was achieved by bioluminescence imaging. Animals were injected intraperitoneally with 180 mg/kg luciferin potassium salt (Caliper file sciences, Roissy, France) before undergoing anesthesia with sevoflurane in an induction box. Then, 2% sevoflurane in O_2 was continuously delivered *via* a nose cone system in the dark box of the Ivis Spectrum imager equipped with a high sensitivity CDD camera cooled to −95°C (Caliper, USA). Bioluminescence acquisition was performed 15 min after substrate injection. Detection of signal for tumor localization was performed using Living Image 4.2 software (Caliper LS, France). In order to perform Diffuse Luminescence Imaging Tomography (DILT) analyze, a sequence of different images acquired using different emission filters (580 to 680 nm) was realized. Additionally, biodistribution of liposomes was monitored on Day-1 and Day-17 after that mice were administered by IP injection (tail vein) with Lipo-70 , Lipo-120 and Lipo-250 previously tagged with the DIR fluorescent probe (λabs 750 nm, λem 782 nm, Caliper LS, France). After treatment, liposome biodistribution was monitored hourly for 12 h, then daily for up to 25 days. To be sure that observed tumor uptake was not an artifact related to the administration route, biodistribution study was repeated on an independent satellite group after IV injection and monitoring over 47 days. Tumor uptake was defined as the ratio between fluorescent signal emitted from the tumor previously defined as the region of interest (ROI) by previous bioluminescence analysis, and the fluorescent signal emitted from the whole body. Blood vessel density was evaluated using the AngioSens kit (Fluoptics, Perkin Elmer, France). Additionally, whole-body exposure was determined by calculating the respective Area Under Curves (AUCs) obtained when monitoring total fluorescence throughout time after injecting mice with Lipo-70, Lipo-120 and Lipo-250.

In Vivo Antitumor Activity

Four-week old female Swiss, nude mice ($n=8$ per group, Janvier, France) were inoculated in mammary fat pad with 1×10^5 MDA231 cells. Matrigel (60%) was used as graft matrix. Ten days after inoculation (i.e., when tumor volume reached about 1 mm^3), mice were randomly allocated into four groups (control, Lipo-70, Lipo-120 and Lipo-250, 8 mice per condition). The mice were injected *via* the tail vein and were treated in an equi-dose fashion with 30 mg/kg and 40 mg/kg respectively for 5-FU and d-Ino with Lipo-70, Lipo-120, with Lipo-250. Animals were treated on a once-a-week basis for 5 consecutive weeks. Tumor size, localization and metastasis spreading were measured thrice a week by bioluminescence following the same procedure than described previously.

RESULTS

Liposomes Characteristics

Different mean diameters (i.e., 70, 120 and 250 nm) were obtained according to the different sonication times. Mean diameter was 72.62 nm (94.3%), 130 nm (100%) and 263.5 (98.9%) for Lipo-70, Lipo-120 and Lipo-250, respectively (data not shown). Incorporation-rates of 5-FU as determined by HPLC was 19, 27 and 37%.for Lipo-70, Lipo-120 and Lipo-250 respectively .and encapsulation rate of d-Ino was 13.4, 23 and 26% for Lipo-70, Lipo-120 and Lipo-250 respectively.

In Vitro Antiproliferative Assay

In MDA MB231 cells, IC50 values were 10±2 μM, 5.7± 1 μM and 8±0.5 μM for Lipo-70, Lipo-120 and Lipo-250 respectively. IC50 for free 5-FU associated with d-Ino and FA was 6±1.6 μM. No significant difference was observed between the values.

Immunofluorescence Staining

Microscopy-Fluorescence showed that liposomes were internalized into tumor cells, regardless of their respective

diameters. We further showed that liposomes were uniformly distributed in the entire cytoplasm, but not in the nucleus (data not shown). Liposome size had an impact on the kinetic of the internalization: Lipo-70 proved to be internalized faster (i.e., one hour of exposure), whereas for the bigger liposomes, 4 h were necessary to the cells to uptake the particles (Fig. 1).

Biodistribution Studies in Mice

Upon first injection, we observed during the first 24 h a slow distribution of the liposomes throughout the body, regardless of the size. Transient liver uptake was observed 4-5 h after administration. At Day-7, the smaller were the liposomes, the higher was the tumor uptake. Tumors were the tissues emitting the strongest fluorescent signals. Conversely, in mice injected with Lipo-250 liposome was apparently still distributed throughout the whole body, in addition to the tumor tissue (Fig. 2). Second injection of the three different batches of liposomes was realized 2 weeks after the first injection, when tumors were bigger with a more dense vascularization network as visualized by the Angiosense staining. We observed a strong correlation between tumor size and vascularization density ($r^2 = 0.837$). Liposome uptake was always greater when mice displayed bigger tumors with a more dense vascular network, regardless of the liposome sizes. Still, we observed a greater difference in tumor-uptake between mice treated with Lipo-70, and larger liposomes when tumors were bigger and more vascularized (Fig. 3). At study conclusion, a statistical difference in relative tumor uptake was observed between the groups (Lipo-70: 28.2±6.4%, Lipo-120: 16.2±4.5%, Lipo-250: 17.7±3.2%, $p<0.05$, One Way-Anova with Duncan's multiple comparison testing). Biodistribution study was repeated after I.V injection to confirm that the initial IP route did not lead to an artifactually elevated tumor uptake because drug was injected in a similar area than the lower mammary fat pad used when grafting the tumor cells. Data confirmed the higher tumor uptake of small liposomes as compared with larger ones. Midterm analysis (i.e., Day-30) showed that mean tumor uptake was 14.5%, 10.5% and 12.7% for Lipo-70, Lipo-120 and Lipo-250, respectively. At study conclusion (i.e., Day-44), mean tumor uptake was 25.3%, 18.4% and 16.5% for Lipo-70, Lipo-120 and Lipo-250, respectively. A significant difference in tumor uptake was found between the groups ($p<0.05$, One-Way Anova with Duncan's multiple comparison testing). Of note, the increase of tumor uptake throughout time has to be considered as relative, because overall fluorescent signaling decreased from the time the liposomes were injected into the animals (Fig. 3, insert). Monitoring of the total fluorescence in mice showed that two separate injections of 30 mg/kg of 5-FU as DIR-liposomes led to different exposure levels, the second injection leading to lower exposures, regardless of the liposome size. Area under the Curve (AUC) was 45.3, 80.4 and 64.8×10^6 p/s.days^{-1} for Lipo-70, Lipo-120 and Lipo-250 after the first injection and 35.1 (i.e., −22.5% as compared with first injection) , 51.9 (−35.4%) and 51 (−21.3%) p/s.days^{-1} after the second injection.

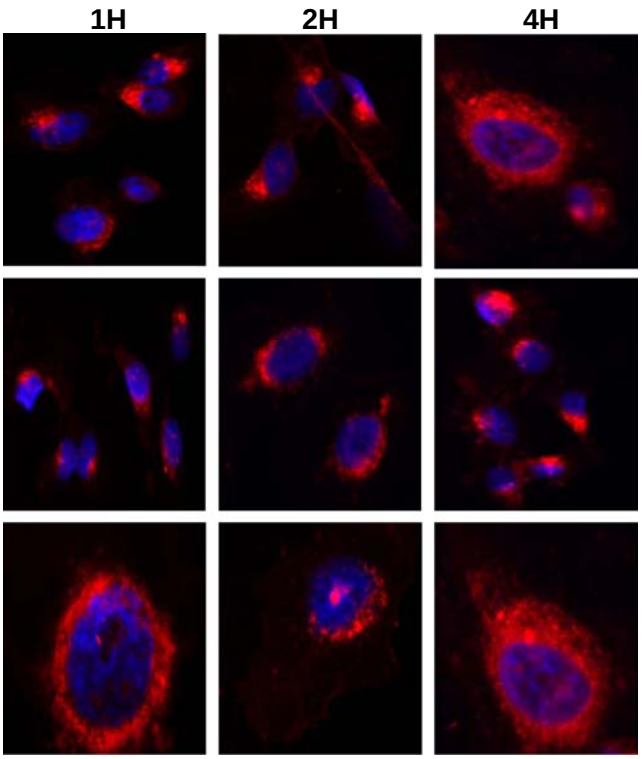

Fig. 1 Penetration in MDA MB 231 breast cancer cells, after 1H, 2H and 4H exposure with L-120 liposomes and Immunofluorescence staining. The cells exhibited red color in the cytoplasm region with a diffuse distribution and a blue fluorescence of DAPI from the nucleus, suggesting that after cellular uptake, DIR liposomes are mainly distributed uniformly in the entire cell cytoplam and not in the nucleus.

Antitumor Efficacy

Marked differences in tumor growth were observed among the different groups. Mean tumor volumes at midterm analysis (i.e., Day-38) expressed as 10^6 p/s were 3.04±2 (Lipo-70), 2.28±1 (Lipo-120), 5.88±0.9 (Lipo-250) and 3.62±3.2 (control). However no significant difference was found ($p=0.625$, one-way Anova). At study conclusion (i.e., Day-65); tumor volumes were 11.2±9 (Lipo-70), 14.25±5.7 (Lipo-120), 36±21 (Lipo-250) and 23.2±5.5 (control) (Fig. 4). This difference was found to be statistically significant ($p<0.05$, one-way Anova with Duncan's multiple comparison testing). Survival at 60 days was 100% (Lipo-100), 85% (Lipo-70), 60% (Lipo-250) and 75% (control) (Fig. 5). 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).

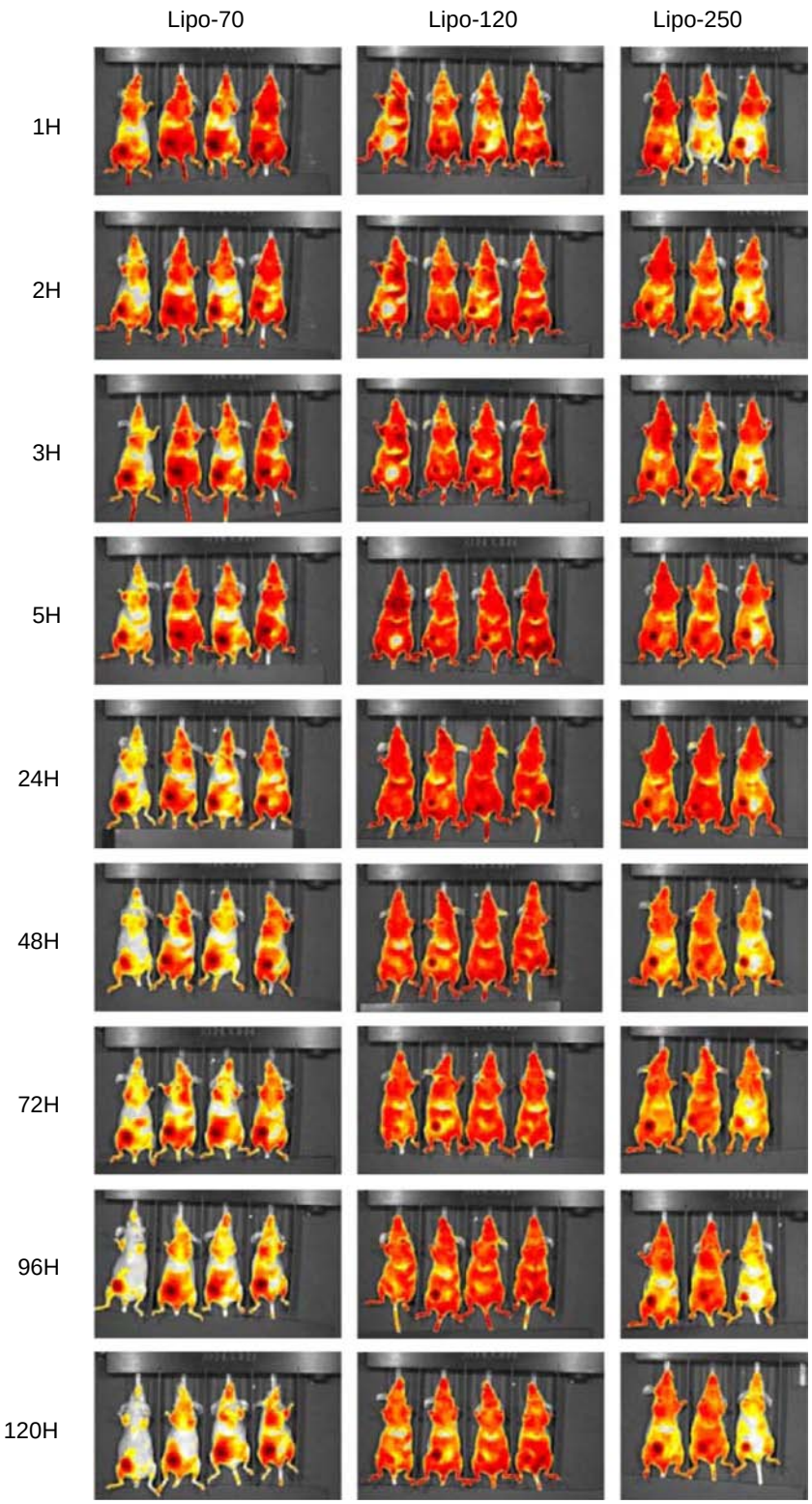

Fig. 2 Monitoring of fluorescence emission throughout time in mice administered with DIR-labelled liposomes of three different sizes.

DISCUSSION

Previous studies have shown that modulated stealth liposomal 5-FU may provide a clinical benefit in various tumor models, particularly against fluoropyrimidine-resistant colorectal tumors (e.g., SW620, LS174) (17) but not against MDA MB231 breast cancer cell lines (13). In this study we intentionally used again this canonical, highly resistant MDA breast cancer model. Drug resistance is indeed a major issue at the bedside and there was little interest to test the impact of a re-

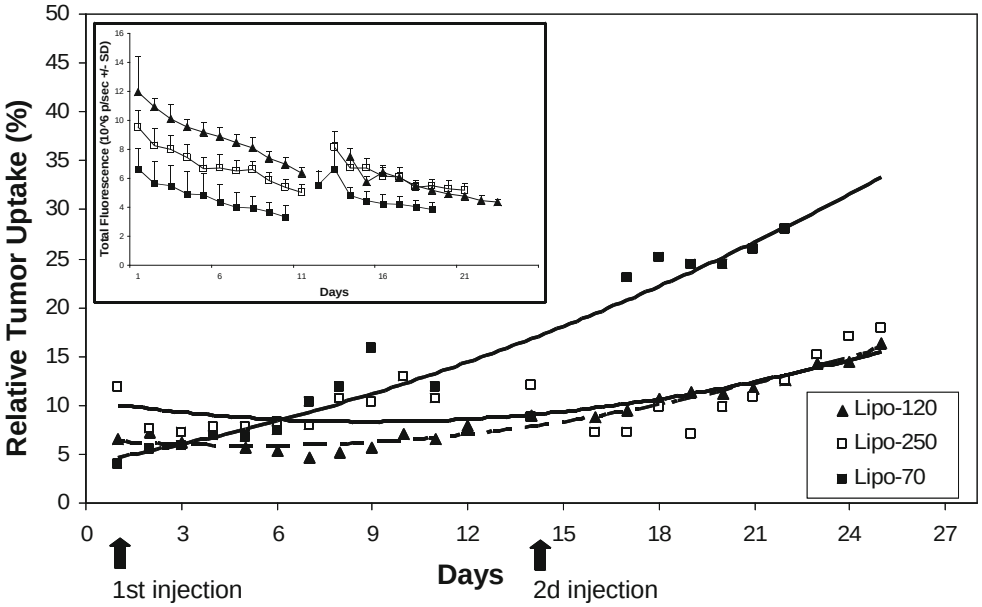

Fig. 3 Relative tumor uptake according to particle's size after I.P. injection of DIR-labelled liposomes. A significant difference was observed between the groups ($p < 0.05$, One-Way Anova). *Insert*: kinetics of the total fluorescence after I.P. injection of DIR-labelled liposomes.

designed liposomal formulation in a sensitive model. This is why in this new study, we did focus specifically on the most resistant model to check the hypothesis that designing smaller stealth particles could help to reverse the initial resistant phenotype. Liposome formulation previously developed was characterised by size comprised between 100 and 120 nm. Nanoparticle size is directly involved in the passive targetting of tumors. Nanoparticles with a diameter between 100 and 200 nm have been shown to have a higher rate of uptake than do those with diameters higher than 300 nm or less than 50 nm. Moreover, the size impacted the encapsulation rate, particularly for hydrophilic drug like 5-FU. In our experiments, encapsulation rate was 19% for the smaller liposomes (i.e., 70 nm diameter), a value that was found to be acceptable, regarding the low-cost of 5-FU and the fact that volumes to be injected in animals to reach 30 mg/kg of 5-FU delivered as Lipo-70, Lipo-120 and Lipo-250 respectively were in agreement with animal ethics. However, reducing further the diameter could have led to smaller encapsulation rates, thus requiring larger volumes of the liposomal suspension to be injected, therefore unmeeting the guidelines of animal experiments. When considering solid tumors, EPR effect provides an opportunity for a more selective delivery of nanoparticles (18). For an optimized EPR effect, small size particles ($\leq$ 100 nm) with high stability in the bloodstream (e.g., stealth liposomes), are expected to diffuse by passive convective transport through the tumor capillaries (19–21). Tumor capillaries are relatively permeable indeed, and leaking through gaps in the vasculature can occur for plasma proteins and other macromolecules such as drug-loaded liposomes, provided that

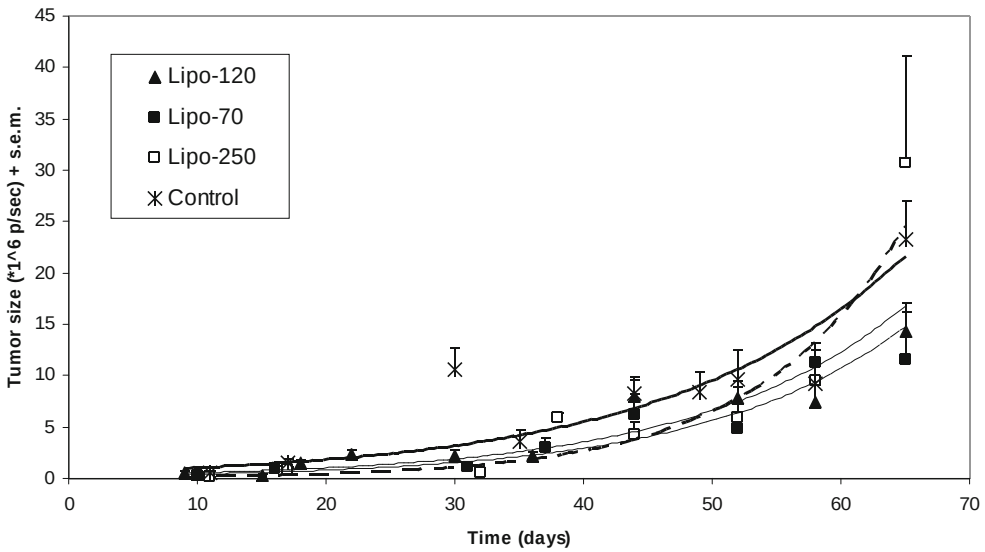

Fig. 4 Comparative tumour growth in mice. Animals were administered I.V. for once a week, over 5 consecutive weeks with each of the following: carbonate, or 30 mg/kg liposomal 5-FU administered as Lipo-70, Lipo-120 or Lipo250 batches. A statistical difference was observed between the groups at study conclusion ($p < 0.05$, One Way Anova).

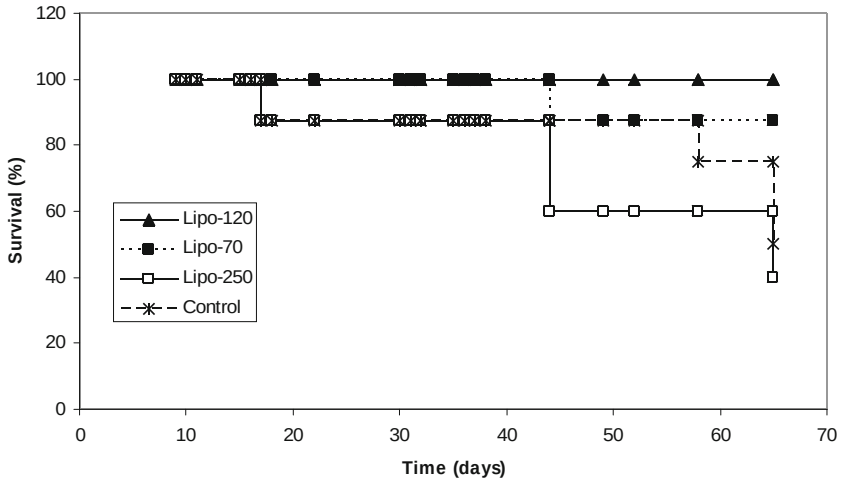

Fig. 5 Survival in tumor-bearing animals treated with carbonate, or 30 mg/kg of equivalent 5-FU delivered through different liposome sizes (ie, Lipo-70, Lipo-120 and Lipo-250).

their diameter is suitable for leaking out the blood vessels. Here, we investigated to what extent different size in liposomes would change their distribution and selectivity towards tumor tissues, thereby achieving greater antitumor efficacy eventually, including in the MDA231 breast cancer model previously identified as highly resistant to 5-FU. *In vitro* studies showed that differences in liposome diameters had no impact on sensitivity, because no statiscal difference was observed in the resulting IC50s.

Monitoring the distribution over several days showed that little liver uptake occurred, regardless of the diameter of the liposomes, much probably because of the pegylation of the particles providing a stealth behavior once in the body. Similarly, tumors were the tissues which concentrated most of liposomes, regardless of the size of the particles. However, this phenomenon was significantly more marked with small liposomes because higher concentrations in tumors were observed with Lipo-70, an observation consistent with the hypothesis that smaller liposomes will leak more easily than larger ones. Of note, it is not possible to claim that the presence of high levels of fluorescence is a demonstration of the accumulation of the active drugs at the tumor level, but only that DIR-labeled lipids were present. Biodistribution studies demonstrated first an inverse-correlation between tumor uptake and liposome size, and secondly a correlation between tumor uptake and tumoral vascularisation. This suggests that encapsulated drugs exhibit a higher specificity against big tumors as compared with smaller ones or secondary metastases. Monitoring of the total fluorescence in mice showed that exposure levels were lower after the second injection. This observation is consistent with the ABC (accelerated blood clearance) phenomenon described when liposomes are injected repeatedly over small intervals as we did (22,23). Finally, the smaller was the liposome, the higher was the efficacy in tumor-bearing mice, an observation fully consistent with the significant differences in tumor uptake we evidenced previously. A significant difference in size was evidenced at study conclusion, the smaller the liposome, the greater the reduction in tumor volume. However, impact of liposome size on survival could only be characterized as a trend toward longer survival in animals treated with smaller liposomes. Of note, in our experiments mice treated with lipsomes of 250 nm in diameter had shorter survival as compared with any other group, a finding consistent with the fact that at study conclusion, big liposomes showed indeed no antiproliferative activity as compared with untreated animals. Survival in mice treated with Lipo-250 was even shorter than survival in the control group. Despite the absence of changes in carcass weight, this could be due to toxicities leading to multiple organ failure in animals administrated with big liposomes, although further studies should be performed to elucidate this point. Overall, our data suggest therefore that antitumor efficacy and survival can be improved by designing smaller liposomes, because data show that the smaller are the particle, the better was the outcome in tumor-bearing mice. Additionally biodistribution studies suggest that greater tumor uptake achieved with smaller liposomes could explain the gain in efficacy observed next in tumor-bearing animals. Still, even when using small liposomes, impact in term of tumor growth was modest, much probably because of the unfavorable molecular profile of the MDA231 cells. Poor TP/DPD ratio, as evidenced by others, makes indeed this model highly resistant to fluoropyrimidine drugs (13). Despite an increase in overall survival and a significant 62% reduction in tumor growth at study conclusion, Lipo-70 failed in totally reversing the resistant phenotype, as highlighted by the elevated IC50 values recorded *in vitro*, and the fact that only a slow-down in tumor growth was achieved in animals. In this respect, the issue of resistance to 5-FU with MDA-231 cells is probably not solely related to bad pharmacokinetics and poor delivery that could be addressed by designing small nanoparticles, but as well to some insufficient inhibition of the target once the drug has reached the tumor and unfavorable molecular profile. However, despite the only relative gain in

efficacy we observed by designing small stealth liposomes, the data presented here strongly support the hypothesis that blood vessel density and particle size are both critical parameters to better target solid tumors through the EPR effect (4,24,25).

CONCLUSION

Achieving better specificity with anticancer agents is a rising concern in experimental and clinical oncology (26). Our experimental data support the hypothesis that when designing liposomes, special attention must be paid to particle size. Marked differences were observed indeed in antiproliferative efficacy recorded between small (i.e., 70 nm) and big liposomes (i.e., 250 nm). Encapsulating drugs into nanoparticles is therefore a promising strategy because passive targeting can be achieved through leaky blood vessels in the tumor surroundings, provided that particles have the appropriate diameter.

ACKNOWLEDGMENTS AND DISCLOSURES

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