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Research Paper

Development of Stealth Liposome Formulation of 2′-Deoxyinosine as 5-Fluorouracil Modulator: *In Vitro* and *In Vivo* Study

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Purpose. The aims of this study were to develop a stealth, pegylated liposomal formulation of 2′-deoxyinosine (d-Ino), a 5-fluorouracil (5-FU) modulator, to evaluate its efficacy *in vitro* and in tumor-bearing mice, and to study its pharmacokinetics in rats.
Method. After designing a pegylated liposome encapsulating d-Ino (L-d-Ino), we evaluated its efficacy as 5-FU modulator *in vitro*. Antiproliferative assays, thymidylate synthase (TS) inhibition, and apoptosis studies were carried out to check whether an optimization of 5-FU action was achieved on the 5-FU-resistant SW620 cell line. Animal pharmacokinetic and *ex vivo* studies were next performed to confirm that L-d-Ino displayed a slower plasma elimination pattern than free d-Ino. Finally, effects on tumor growth of L-d-Ino + 5-FU combination was evaluated in xenografted mice.
Results. We developed a stable, sterile, and homogenous 100-nm population of pegylated liposomes encapsulating 30% of d-Ino. Liposomal d-Ino exhibited a strong potential as 5-FU modulator *in vitro* by enhancing TS inhibition and subsequent apoptosis induction, while displaying a better pharmacokinetic profile in animals, with a near seven times clearance reduction as compared with the free form. When used in tumor-bearing mice in combination with 5-FU, our results showed next that the association led to 70% of tumor reduction with a doubling median survival time as compared with untreated animals, whereas 5-FU alone was ineffective.
Conclusion. Our data show that liposomal d-Ino, through an optimized pharmacokinetic profile, displays a potent effect as fluoropyrimidines modulator, both *in vitro* and in xenografted mice. Besides, we showed here that it is possible to reverse a resistant phenotype to 5-FU, a major drug extensively described in clinical oncology.

KEY WORDS: 2′-deoxyinosine; 5-FU; liposome; pharmacokinetics; thymidylate synthase; xenografts.

INTRODUCTION

Fifty years after its synthesis, prodrug 5-fluorouracil (5-FU) remains a mainstay in the treatment of colorectal cancer. However, because its overall response rate does not exceed 20–30% in monotherapy (1), improving 5-FU efficacy is still a major concern of today's oncology. We showed previously that 2′-deoxyinosine (d-Ino) enhances the cytotoxicity of 5-FU by increasing thymidine phosphorylase (TP) activity, the tumoral enzyme responsible for the near-direct conversion of the drug toward anti-thymidylate synthase (TS) 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) metabolite (2–4). d-Ino is a precursor of TP cofactor deoxyribose 1-phosphate, whose use proved to enhance 5-FU antiproliferative activity on different models (5–8).

In vitro, d-Ino proved to increase the sensitivity to 5-FU of various colorectal cell lines and, *in vivo*, to enhance tumor growth reduction in xenograft-bearing mice treated with 5-FU. However, because of its unfavorable pharmacokinetic profile, we found that up to 3.2 g/kg of d-Ino had to be administered daily to the animals to overcome extensive erythrocytic catabolism by purine nucleoside phosphorylase or PNP (9–11). Such a requirement dramatically hindered any further use of d-Ino *in vivo*, at least in its free form, in spite of striking achievements *in vitro*.

Liposomes are self-closed, spherical vesicles, in which a phospholipid bilayer encapsulates drug-containing aqueous space, thus allowing some protection from catabolism. To date, liposomes are the only drug delivery systems applied to cancer chemotherapy (12). The purposes of the present study were to develop a new, stealth liposomal formulation of d-Ino, to spare it from the catabolic action of PNP, and to allow a better handling as an *in vivo* modulator of 5-FU.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Overexpressing TS, 5-FU-resistant, SW620 (a.k.a. CCL227) human colon carcinoma was kindly provided by

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Dr. Gerard Milano, Centre Antoine Lacassagne (Nice, France). Cells were maintained at 37°C in RPMI supplemented with 10% fetal calf serum, 1% glutamine, 10% penicillin, 10% streptomycin, and 1% kanamycin in a humidified CO_2 incubator.

Drugs and Chemicals

Egg yolk phosphatidylcholine (PC), phosphatidylglycerol (PG), cholesterol (C), polyethylene glycol (PEG) covalently binded to phosphatidylethanolamine, d-Ino, 5-FU, and 5′dFUR were all purchased from Sigma (St. Quentin, France). Tritiated 5-FU and dUMP (12 Ci/mmol) came from DuPont-New England Nuclear (Boston, MA, USA). Dikalium hydrogenous phosphate (K_2HPO_4) buffer, acetonitrile, ether, and methanol came from CarboErba (Milan, Italy). Dimethyl sulfoxide was purchased from Euromedex (Souffelweyersheim, France). All reagents were of analytical grade.

Liposomes Preparation

Liposomes were prepared following the classic thin film method (13). Lipids were first dissolved in methanol until a clear lipid solution was obtained. Solvent was then removed by rotary evaporation at 37°C (Rotavapor, Büchi, Rungis, France), thus leaving a thin lipid film.

Subsequently, the lipid film was placed under vacuum of nitrogen to remove residual solvent. Hydration of the dry lipid was accomplished by adding 5 ml of the drug solution. The resulting milky suspension was sonicated (20 kHz) for 5 min at 4°C using a Branson Sonifier 250 sonic probe.

To remove the nonincorporated drug, the liposomal suspension was ultracentrifuged at 70,000 × *g* at 4°C for 16 h. The resulting pellet was resuspended in either 10 ml of 10 mM carbonate buffer (pH 7.4) or culture media, depending on the future use (*in vitro*, *ex vivo*, *in vivo*) of the preparation. Liposomes were finally filtered at 0.2 μm (Durapore, Millipore, Molsheim, France) to ensure the sterility of the final preparation. When required, releasing of liposomal d-Ino was next monitored by dialysis. Sampling was performed every 15 min up to 4 h and then every 30 min up to 7 h. The total amount of d-Ino released was determined by UV spectrophotometry at 248 nm.

Polydispersity Study

Diameters and particle size distribution were determined by photon correlation spectrometry using a Correlateur RTG submicron particle analyzer (Sematech, Nice, France). Measurements were performed from a 90° angle at room temperature.

Cytotoxicity Study

Aliquots of SW620 cell suspension (10^4 cells per well) were seeded in 96-well plates that were incubated for 24 h at 37°C in a fully humidified 5% CO_2 atmosphere. Cells were then incubated for 24 h without (control) or with different concentrations of 5-FU alone or combined with 800 μM free or liposomal d-Ino. After 24 h of continuous exposure with gentle shaking, cells were washed with fresh medium and allowed to grow for 48 extra hours in drug-free medium. Cell growth was evaluated using the classic colorimetric 3-(4,5-dimethyltriazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide assay. For the MTT assay, cells were incubated for 2 h in 200 μl of MTT and lysed in dimethyl sulfoxide; viable cells were monitored at 550 nm for the conversion of MTT to formazan. IC_{50} was defined as the 5-FU concentration inhibiting 50% of cell growth as compared with untreated wells.

5-FU Metabolism Study

Monitoring of 5-FU tumoral activation was performed as described previously by radio-high-performance liquid chromatography (radio-HPLC) analysis (10,11). Exponentially growing cells were exposed to 1 μM of tritiated 5-FU, associated or not with 800 μM of either free or liposomal d-Ino. Cells were harvested after 4- and 5-h exposure, lysed in 60% methanol, and cytosol-extracted prior to HPLC analysis with radioactive detection (Flow-On A500, Packard, Issy Les Moulineaux, France).

TS Inhibition Study

Thymidylate synthase activity and inhibition were assessed as described previously (10,11). Exponentially growing cells were exposed to various combinations of 10 μM 5-FU alone, associated with 800 μM of free or encapsulated d-Ino. Inhibition of TS activity was evaluated after 24-h exposure. Cells were then harvested and centrifuged, and the pellet was stored at −80°C until analysis. TS activity was assayed following the standard Roberts (14) method based on the tritiated H_2O release from [^{3}H]-dUMP in the presence of excess of methylene tetrahydrofolate.

Apoptosis Studies

Cells in exponential phase were exposed to 5-FU (20 μM) alone, with free or liposomal d-Ino (800 μM) for 24 h. Cells were harvested; early apoptotic changes and late apoptosis were discriminated by simultaneous staining using Annexin V FITC kit (Euromedex). Cells were treated following the manufacturer's guideline. Flow cytometry analysis was carried out in a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) using CellQuest software. Apoptosis threshold measured in untreated cells was defined as 100%.

Stability Study of d-Ino in Blood: *Ex Vivo* Experiments

Heparinized fresh whole blood withdrawn from anesthetized rats was incubated at 37°C in a shaker with either free or liposomal d-Ino (final concentration, 1 μg/ml). Samples were taken at 0 and then at 30, 60, 120, 180, and 240 min after incubation, centrifuged at 5000 × *g* for 10 min, and the resulting plasma was stored at −20°C until analysis.

Animal Pharmacokinetic Studies

Male Wistar rats were kept anesthetized using O_2/NO_2 gas + isoflurane (TEM, Bordeaux, France) during the whole study. Body temperature was maintained at 37°C using a warming blanket. Animals (n = 6/group) were administered

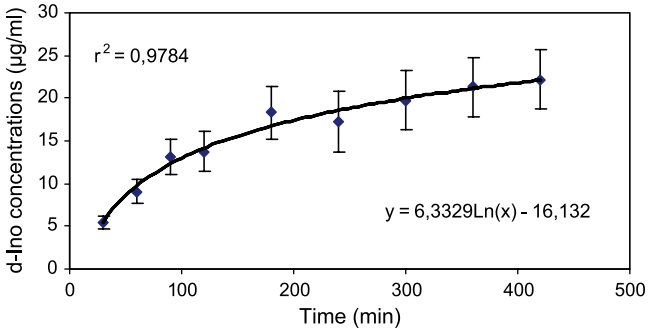

Fig. 1. Monitoring of 2′-deoxyinosine (d-Ino) release from the liposomal form. d-Ino dialysis was measured at 248 nm.

with free (150 mg/kg) or liposomal d-Ino (9 mg/kg) by intraperitoneal (i.p.) injection. Sampling times were as follows: T0, T15, T30, T45, and T60-min. One milliliter of blood was withdrawn from jugular vein on heparinized tubes, and the plasma was isolated by centrifugation at 5000 × *g* for 10 min. Plasma samples were stored at −20°C until analysis. Animal study was performed following animal welfare guidelines (15).

d-Ino Assay in Plasma

Four hundred microliters of plasma was placed in glass tubes. A 100-µl volume of internal standard (5′dFUr, 1 mg/ml) and 7 ml of isopropanol/ether (25/75, v/v) were added successively. The tubes were vigorously vortexed for 1–2 s, shaken for 10 min at room temperature, and finally centrifuged at 12,000 × *g* at 5°C. The upper organic layer was placed in glass tubes and dried under vacuum at 37°C.

The residues were reconstituted in 120 µl of mobile phase. Samples were vortexed for 15 s. If required, an extra-centrifugation step was performed using a microcentrifuge (12,000 × *g*, 5°C) to clear the solution. The sample was next transferred into a microvial, and 100 µl was finally injected into the HPLC system.

HPLC Analysis

Detection and quantification of d-Ino in plasma samples were carried out by HPLC (HP-1090, Hewlett-Packard, Issy Les Moulineaux, France). Separation was performed at ambient temperature on a Lichrosphere 5-µm particle, 250 × 4 mm, C18 column (Waters, Yvelines, France). The mobile phase consisted of 0.1 M K_2HPO_4 and methanol. A linear gradient elution program was applied as follows: 4–25% methanol, from 0 to 23 min. Chromatographic separation was performed at 1 ml/min and monitored at 248 nm (HP-1100 UV detector, Agilent, Paris, France). Data collection and analysis were performed using Chemstation software (Agilent).

Efficacy Study

Twenty-eight male nude (nu/nu) mice (Iffa Credo, L'Abresle, France) were subcutaneously (s.c.) transplanted on the left flank with 10^6 SW620 cells. Ten days after transplantation, tumors were assessable for measurement, and the animals were randomly separated in four different groups (n = 7/group). Treatment was performed intraperitoneally once daily on three consecutive days as follows: control (carbonate buffer), 5-FU alone (50 mg/kg), 5-FU (50 mg/kg) + free d-Ino (50 mg/kg), and 5-FU (50 mg/kg) + L-d-Ino (50 mg/kg). Tumor growth was monitored three times a week using a caliper as described previously (10,11). Animal weights were controlled as a surrogate marker of treatment-related toxicity. Mice care was in agreement with animal welfare guidelines (15).

Pharmacokinetic and Statistical Analysis

Calculations of plasma clearances and area under curve (AUC) were performed using the Apis 4.16 version software (MIPPS, Marseilles, France) using formulas extensively described elsewhere (16). For both *in vitro* and *in vivo* experiments, differences between mean values were evaluated using either one-way ANOVA with Tukey test or Student–Newman–Keuls test, according to data distribution. A p value of <0.05 was regarded as statistically significant (Sigma Stat Software, SPSS, Essen, Germany).

RESULTS

d-Ino Incorporation and Release

d-Ino was encapsulated into vesicles composed of egg PC/PG/CHOL/PEG in the molar ratio of 43:33:4:1 (mol/mol). Mean encapsulation rate was 30 ± 4% (n = 5), giving an encapsulation efficiency of 27.2 mol of d-Ino per mole of lipid. Drug release from liposomes was monitored by dialysis, and the release profile was described by a logarithmic curve following $c(t) = 6.33 \times \ln(t) - 16.13$. A maximum release of 60% was reached after 4 h of incubation (Fig. 1). Release steady state (93%) was finally observed after 6.5 h of incubation.

Polydispersity Study

Laser light scattering showed a single vesicle population of 114 ± 12 nm. No difference in size was found before and after filtration (data not shown).

Modulation of Antiproliferative Activity

Associating liposomal d-Ino to 5-FU significantly increased SW620 sensitivity ten times (Fig. 2). After associating 5-FU with

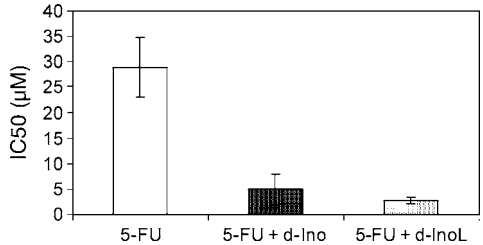

Fig. 2. Modulation of 5-fluorouracil (5-FU) cytotoxicity by free (black bar) or liposomal (grey bar) d-Ino. Cells were exposed for 24 h to 5-FU alone in combination with 800 µM d-Ino. Cell viability was measured by 3-(4,5-dimethyltriazol-2-yl)-2,5-diphenyltetrazolium 48 h after removing the drugs. Bars: standard deviation (SD).

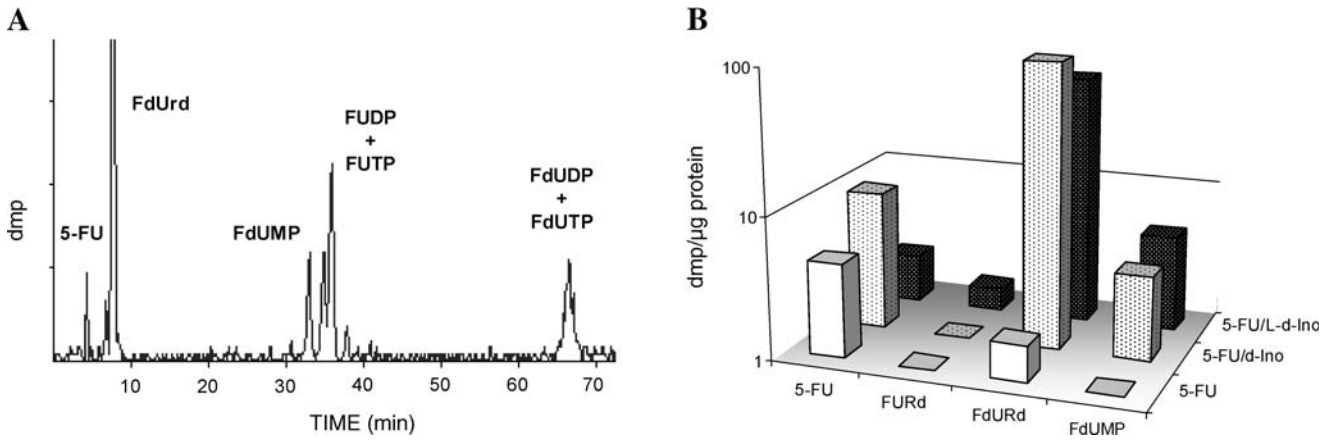

Fig. 3. Monitoring of 5-FU intratumoral activation by radio-high-performance liquid chromatography (radio-HPLC). (A) Typical chromatogram of 5-FU activated following the DNA pathway. (B) Comparison of the activation patterns according to the combination regimen. SW620 cells were exposed for 4 and 5 h to 1 μM of tritiated 5-FU alone (white bars) or associated with either free d-Ino (grey bars) or L-d-Ino (black bars). Values are from one representative experiment. Furd: fluorouridine; FdURd: fluorodeoxyuridine; FdUMP: fluorodeoxyuridine monophosphate; FUDP + FUTP: fluorouridine diphosphate and triphosphate; FdUDP + FdUTP: fluorodeoxyuridine diphosphate and triphosphate.

free and liposomal d-Ino, IC_{50} was reduced from 29 ± 6 down to 4.9 ± 3 and 2.75 ± 0.8 μM, respectively ($p < 0.05$, one-way ANOVA with Tukey test). Conversely, no statistical difference was detected between free and encapsulated d-Ino in our experimental conditions ($p > 0.05$).

5-FU Metabolism

Typical chromatogram of modulated 5-FU is displayed in Fig. 3A. When used alone, 5-FU was activated following the RNA pathway, and no anti-TS FdUMP metabolite was detected in our experimental conditions. Oppositely, combining the drug with either free or liposomal d-Ino triggered the alternative DNA pathway, with subsequent enhanced formation of FdURd and pharmacologically active FdUMP (Fig. 3B). Finally, DNA incorporation of tritiated 5-FU as FdUTP was enhanced by 18 and 32% after combination with free and liposomal d-Ino, respectively.

TS Inhibition

Results of TS inhibition are displayed in Fig. 4. 5-FU alone showed no significant effect on TS activity, whereas adding either free or liposomal d-Ino enhanced TS inhibition in the SW620 line. TS activity was inhibited by up to 95 and 65%, respectively, after 24-h exposure ($p < 0.05$, one-way ANOVA with Newman–Keuls test).

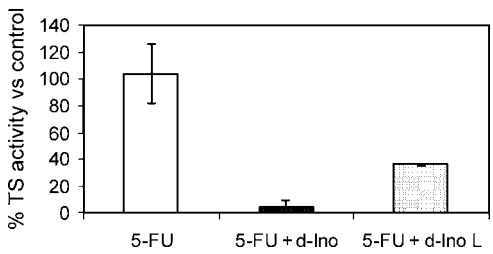

Fig. 4. Enhancement of 5-FU-induced thymidylate synthase inhibition by free (black bar) or liposomal (grey bar) d-Ino. Cells were exposed for 24 h to 10 μM 5-FU alone or in combination with 800 μM d-Ino.

Apoptosis Studies

A greater induction of both early and late apoptosis was observed in SW620 exposed to 5-FU combined with free or liposomal d-Ino, as compared with 5-FU alone (Fig. 5). Early apoptosis was increased by 273 and 139% after associating 5-FU to free and liposomal d-Ino, respectively. Similarly, late apoptosis was enhanced by 161 and 211% when 5-FU was combined to free and liposomal d-Ino, respectively ($p < 0.05$, one-way ANOVA).

Ex Vivo Experiments

Degradation curves of free and liposomal d-Ino in whole blood are displayed in Fig. 6. d-Ino levels were up to five times higher when incubated as a liposomal form, as compared with free d-Ino. Besides, after 1-h incubation in plasma, free d-Ino was totally catabolized, whereas its encapsulated counterpart was still detectable after up to 3 h of incubation.

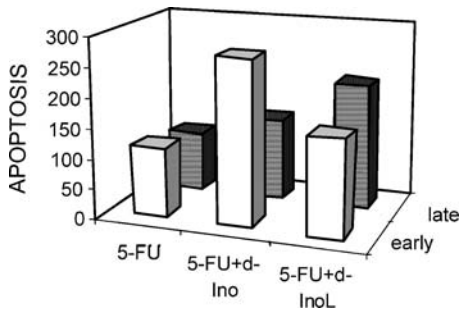

Fig. 5. Enhancement of 5-FU-induced apoptosis by free or liposomal d-Ino. SW620 cells was exposed to 20 μM of 5-FU alone or combined with 800 μM of d-Ino, either free or liposomal. Early (white bars) and late (black bars) apoptosis was detected by IP/Annexin V double staining with subsequent flow cytometry analysis.

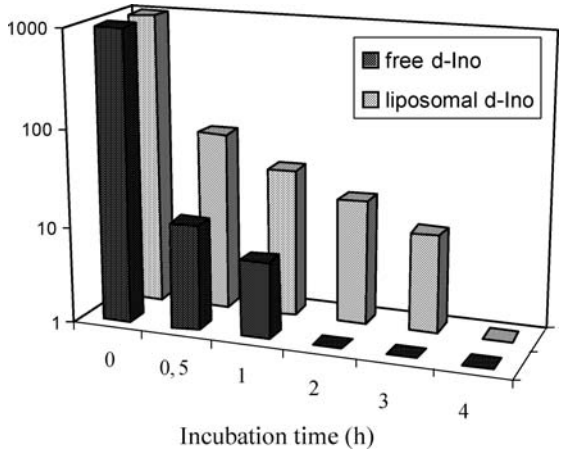

Fig. 6. Ex vivo study of d-Ino catabolism in rat whole blood. The degradation curves of free and liposomal d-Ino were monitored up to 4 h by HPLC analysis.

In Vivo Studies

Free and encapsulated d-Ino were administered at 150 and 9 mg/kg, respectively (dose ratio, 16). No signs of toxicity were observed in animals, regardless of the form we administered. C_{max} were 638 and 185 ng/ml for free and liposomal d-Ino, respectively, with corresponding T_{max} at 15 and 30 min (Fig. 7). Systemic exposure to the drug was assessed by calculating the respective AUCs. AUCs were 338 and 137 ng/ml/h for free and liposomal d-Ino, respectively (ratio, 2.5). Mean plasma clearances of free and liposomal d-Ino were 177 ± 209 and 26 ± 9 l/h, respectively ($p > 0.05$, t test).

Efficacy Studies

Treatment began when tumors became measurable, 10 days after s.c. inoculation of the SW620 cells. No signs of toxicity were observed in animals, regardless of the treatment modalities, and no statistical difference was found in animal weights in the different groups (data not shown). At study conclusion, tumor size was reduced by 33, 47, and 71% ($p < 0.05$, one-way ANOVA) in mice treated with 5-FU alone, associated to free d-Ino, and combined with liposomal d-Ino, respectively (Fig. 8). In concordance with this observation, median survival time was increased from 24 days (control) to 26, 31, and 41 days in mice treated with 5-FU alone, associated to free d-Ino, and to liposomal d-Ino, respectively.

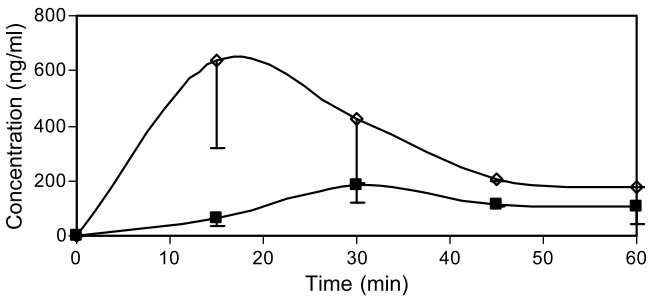

Fig. 7. Pharmacokinetics profile of d-Ino in rats obtained after i.p. administration of free (◊, 150 mg/kg) and liposomal (■, 9 mg/kg) d-Ino. Liposomal d-Ino exhibited a seven times slower plasma clearance as compared with its free counterpart ($p < 0.05$).

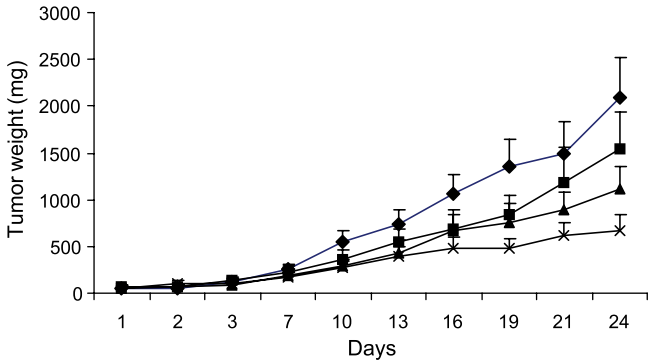

Fig. 8. Effects of combination 5-FU + L d-Ino on SW620 tumor growth in nude mice. Animals (n = 7/group) were subcutaneously transplanted with SW620 tumoral cells and administered for three consecutive days with either of the following: ♦, carbonate (daily i.p.); ■, 5-FU (50 mg/kg, daily i.p.); ▲, 5-FU (50 mg/kg, daily i.p.) + d-Ino (50 mg/kg, daily i.p.); ×, 5-FU (50 mg/kg, daily i.p.) + L-d-Ino (50 mg/kg, daily i.p.). Tumor size was measured three times a week using calipers; bars: SD.

DISCUSSION

5-Fluorouracil antiproliferative activity depends on its intracellular conversion to anti-TS FdUMP (17,18). FdUMP direct formation is dependent on TP, the first of a cascade of enzymatic steps known as the DNA pathway (19,20). Still, because of the lack of TP cofactor within tumor cells, many reports have shown that 5-FU was instead mainly converted through the alternative RNA pathway with possible indirect formation of active FdUMP (20,21). Previous studies focused on the use of ribose donors such as d-Ino or ethoxyuridine (10,11,22,23) that can enhance the rate of 5-FU direct conversion to FdUMP through TP, with subsequent increase in drug efficacy. Precisely, we have shown that using d-Ino greatly increased TP activity, FdUMP formation, and, subsequently, TS inhibition in various tumor cell models. As a result, d-Ino led to a significant increase of cell sensitization and apoptotic induction in tumor cells exposed to fluoropyrimidines. *In vivo*, d-Ino combined with 5-FU proved as well to optimize tumor reduction in xenograft-bearing mice (10,11). Still, because d-Ino was likely to be catabolized by erythrocytic PNP, extremely high doses (up to 1.6 g/kg i.p. twice daily) were used, thus hindering the clinical perspective of using any further this modulator.

In this respect, we studied here a new encapsulated formulation of d-Ino, designed to bypass its erythrocytic catabolism. Liposomal formulation was chosen to entrap d-Ino, using negatively charged membrane that can reduce liposome aggregation to increase stability in suspension (24). Small unilamellar vesicles were chosen in this study because several papers previously reported that 100- to 200-nm liposomes were more likely to accumulate within solid tumors (25). Besides, other reports have shown that the capillary permeability of the endothelial barrier in newly vascularized tumor was significantly higher than that of healthy tissue, thus ensuring a better tumoral uptake (25,26). Additionally, inclusion of cholesterol modulates liposome rigidity and reduces

serum-induced instability caused by binding of serum protein to the membrane (27,28). Finally, adding PEG covalently bound to phosphatidylethanolamine on liposome membrane ensured a better distribution of d-Ino by reducing macrophage uptake in the liver (28–31).

Encapsulation rate of d-Ino (30%) was consistent with data from literature reporting encapsulation of chemically similar compounds and was high enough to consider its use in association with 5-FU. Similarly, reaching a 60% release at 4 h was considered as acceptable to us, with regards to the kinetics of the metabolization pathway of 5-FU that showed that an activation of 5-FU by TP in a 3- to 6-h period was associated indeed with optimized antitumoral efficacy (10, 11, 32).

The cytotoxic activity of liposomes entrapping d-Ino and associated with 5-FU was tested next on SW620 human adenocarcinoma cell line. The SW620 line was chosen in this study because it overexpressed TS and therefore is highly resistant to 5-FU (33). Because overexpression of TS is a common mechanism of resistance in cancer patients, and that modulating fluoropyrimidine drugs through the DNA pathway should increase the formation of anti-TS FdUMP, this cell line seemed therefore to be a suitable model to assess the efficacy of our new formulation. Associating 5-FU with either free or liposomal d-Ino led to a similar six times increase in cell sensitivity, thus showing that encapsulation process did not alter the efficacy of this modulator. Besides, it was checked as well that empty liposomes did not display cytotoxic effect on SW620 cells (data not shown).

We showed next that using either free or encapsulated d-Ino switched the metabolic pathway of 5-FU from RNA to the DNA route, with subsequent increase in active FdUMP formation and final DNA incorporation. These observations strongly reinforce the hypothesis that d-Ino, as a cofactor precursor, enhances tumoral TP activity, thus triggering the DNA pathway after tumoral uptake.

As a pharmacological endpoint, we also checked whether combining 5-FU with liposomal d-Ino would result in an optimized TS inhibition. Results showed indeed that TS activity was significantly reduced when 5-FU was associated with either free or liposomal d-Ino, thus confirming that a better activation of the drug toward anti-TS FdUMP had occurred.

Next, we measured apoptosis induction in SW620 exposed to either 5-FU alone, associated with free, or liposomal d-Ino. In concordance with the previous observations, our data confirmed that combining the drug with liposomal d-Ino did result in a sharp increase in both early and late apoptoses.

In vivo, i.p. administration of free and liposomal d-Ino was performed to determine whether the encapsulated drug displayed indeed slower plasma elimination pattern. Liposomal d-Ino was administrated intraperitoneally because this proved to be a more efficient way to ensure a proper distribution of liposomes (34,35), probably via the lymph node in the abdominal cavity (36).

Pharmacokinetic experiments were performed in rats rather than in nude mice to allow multiple sampling from the same subject, thus lowering the total number of animals to be used and complying with animal welfare guidelines (15).

Liposomal d-Ino was administered at doses as low as 16 times as compared with free d-Ino. Yet, the difference observed between the AUCs of free and liposomal d-Ino was only 2.7. This discrepancy strongly suggests that, considering the differences in dosing, liposomal d-Ino actually leads to higher plasma exposure. Such a difference in exposure could result from either enhanced bioavailability of liposomal d-Ino when given intraperitoneally or reduced clearance once the drug has reached the general circulation. This second hypothesis was retained because calculating the plasma elimination of free and liposomal d-Ino showed that encapsulated d-Ino displayed indeed a near seven times slower clearance than its free counterpart, most probably because of a reduced erythrocytic catabolism. This hypothesis was strongly reinforced by studying the *ex vivo* degradation profiles of d-Ino, which confirmed that the encapsulated form was significantly less catabolized in whole blood than free d-Ino. Importantly, we also showed that total plasma clearance of liposomal d-Ino displayed a markedly smaller interindividual variability than free d-Ino (36 vs. 118%). Although the precise mechanisms of this improved variability remain to be fully investigated, one can assume that L-d-Ino is less affected by erratic PNP activities (9), through a reduced erythrocytic catabolism.

Finally, efficacy studies were undertaken in the classic ectopic tumor-bearing mice model to determine whether low dose of liposomal d-Ino improved indeed 5-FU activity. A significant tumor size reduction (75%) was achieved when combining 5-FU with liposomal d-Ino. Conversely, only relative reduction (−40%) was reached in mice treated with the combination 5-FU + free d-Ino, most probably because of its unfavorable pharmacokinetics profile as shown previously in rats, and 5-FU alone was ineffective on tumor growth. This last observation is fully consistent with the fact that SW620 cells are resistant to fluoropyrimidine drugs. Because we showed *in vitro* that d-Ino, by triggering the DNA pathway, yielded high levels of FdUMP in tumor cells with subsequent optimized TS inhibition, it is much likely that the reversion of resistance we achieved using L-d-Ino was a result of an enhanced anti-TS effect in xenografts. Finally, median survival time of animals treated with this new combination was doubled, as compared with mice treated with standard therapy. Interestingly, the level of antitumoral optimization reached in this study after using 50 mg/kg of L-d-Ino combined with 5-FU was in the range of the ones achieved by using 3.2 g/kg of free modulator in former studies (10,11). Such a difference in dosing (ratio, 64) to achieve similar effects highlights the importance of controlling and improving pharmacokinetic profiles of drugs by means of encapsulated forms.

CONCLUSION

Taken together, our results indicate that 5-FU efficacy can be improved *in vitro* by a modulation strategy using a basically designed liposomal d-Ino, with enhanced TS inhibition and marked sensitization of previously resistant SW620 cell line. In rats, encapsulating d-Ino proved to optimize its pharmacokinetics profile with reduced catabolism, thus permitting to further consider its use *in vivo*, at doses lower than the ones we previously used (10,11). When transposed to an animal model, liposomal d-Ino proved to greatly enhance the antitumoral efficacy of 5-FU, achieving a significant antitumor response in a model that was initially described as resistant to 5-FU. Coencapsulating both 5-FU and its modulator d-Ino in the same vector will have to be

considered in a next step to further optimize drug efficacy both *in vitro* and *in vivo*.

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