Methotrexate loaded SLN prepared by coacervation technique: in vitro cytotoxicity and in vivo pharmacokinetic and biodistribution

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Methotrexate loaded SLNs prepared by coacervation technique: in vitro cytotoxicity and in vivo pharmacokinetic and biodistribution

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Aim: Recently, 'coacervation' has been proposed as a new method to prepare fatty acid solid lipid nanoparticles (SLNs). The aim of this work was to encapsulate methotrexate, a hydrophilic anticancer drug, within SLNs obtained by coacervation, through hydrophobic ion pairing and to evaluate the potential efficacy in in vitro and in vivo breast tumor models of drug-loaded nanoparticles. **Materials & Methods:** Methotrexate-loaded SLN efficacy was evaluated in vitro towards MCF-7 and Mat B-III cell lines (human and murine breast tumor cell lines). Pharmacokinetics of drug-loaded nanoparticles was evaluated in male Wistar rats and biodistribution in a breast tumor model (Mat B-III) in female Fisher rats. **Results:** Drug-loaded SLNs showed an increased cytotoxicity towards MCF-7 and Mat B-III cell lines compared with free drug. After intravenous administration, drug plasmatic concentration was increased and a major drug accumulation within neoplastic tissue was shown when the drug was loaded in SLNs, compared with drug solution alone. Encapsulation of the drug within nanoparticles also increased its oral uptake after duodenal administration. **Conclusion:** SLNs are promising vehicles for the delivery of methotrexate, since an increase of efficacy in vitro and a preferential accumulation in breast cancer in vivo were shown.

Cytotoxic drugs remain the major form of chemotherapy for cancer: they are a diverse class of compounds that treat cancer primarily by being toxic to cells that rapidly grow and divide, such as cancer cells. Cytotoxic drugs are conventionally administered by intravenous bolus or infusion, typically in the form of free drug solutions. Despite the long history of their use and the development of several new multiple drug regimens for improved clinical success, treatment failure is still frequently encountered [1–3].

Conventionally administered cytotoxic agents often extensively and indiscriminately bind to body tissues and serum protein in a highly unpredictable manner. Only a small fraction of the drugs reach the tumor site [4]. This may both reduce the therapeutic efficacy and increase the systemic drug toxicity. Moreover, cancer cells exercise a variety of mechanisms at the cellular level to diminish the toxicity of chemotherapeutic agents they are exposed to. These defensive mechanisms are sometimes categorized as 'cellular' drug resistance [5–7].

Particulate drug carrier systems offer great promise to improve the therapeutic effectiveness and safety profile of this conventional form of cancer chemotherapy. A tumor is often associated with a defective, leaky vascular architecture as a result of the poorly regulated nature of tumor angiogenesis. In addition, the interstitial fluid within a tumor is usually inadequately drained by a poorly formed lymphatic system. As a result, submicron-sized particulate matter may preferentially extravasate into the tumor and be retained there. This is often referred to as 'enhanced permeability and retention' [8]. Moreover, in cytotoxicity studies, drug-loaded nanoparticles are sometimes more cytotoxic to cancer cells than the corresponding free drug. The most probable explanation of this phenomenon is that nanoparticles may carry the drug into the cancer cells by endocytosis, thereby bypassing the P-gP drug efflux mechanism [9,10].

Like other types of drug carriers used for cytotoxic drug delivery, such as polymeric systems and liposomes, solid lipid nanoparticles (SLNs) have the advantages of physical stability, controlled release and easy preparation [11]: they are easier to scale up compared with liposomes and safer compared with polymeric nanoparticles [12]. Recently, in our research group, a new solvent- free technique was developed to produce SLNs of fatty acids by acidification of a micellar solution of their alkaline salts. As pH is lowered, fatty acids precipitate owing to proton exchange between the acid solution and the soap: this process can be defined as 'coacervation' [13]. With this technique, lipophilic drugs can be encapsulated within nanoparticles by allowing their dissolution in micellar solution prior to acidification. It was also demonstrated that, for hydrophilic molecules, hydrophobic ion pairing is an effective strategy to enhance drug encapsulation efficiency within SLNs: a cytotoxic hydrophilic molecule, cisplatin, was encapsulated through hydrophobic ion pairing within SLNs prepared by coacervation [14].

Methotrexate (MTX) is a cytotoxic molecule that is used in the therapy of solid tumors and leukemia, and is preferentially used for the treatment of breast cancer.

It acts as a competitive inhibitor of dihydrofolate- reductase, an intracellular enzyme, which reduces folic acids to tetrahydrofolate cofactors, which are, in turn, key intermediates in

several important biochemical pathways, among which are the de novo biosynthesis of purines and of thymidilate. The lack of reduced folates, purines and thymine in actively proliferating cells, such as those of tumors, leads to a blockage of DNA and RNA synthesis, and eventually to cell death [15].

Since MTX inhibits a key cellular function, it is a very cytotoxic compound, especially towards actively replicating cells. Its cytotoxic activity is exerted not only towards neoplastic cells, but also towards other tissues, thus determining bone marrow depression, inflammation of the upper gastrointestinal tract (mucositis) and severe gastrointestinal disturbance, especially in patients treated at the higher doses now employed to improve its efficacy on some tumors [15].

Due to its high polarity (at the neutral pH of biological fluids it is present mainly in the doubly anionic species), MTX can only cross the cell membrane with difficulty, and is therefore transported into the cells by a specific, high-affinity carrier system [15].

Methotrexate is therapeutically administered by both the oral and the parenteral route. Low doses of MTX (<25 mg/m^2 of body surface) are readily absorbed from the gastrointestinal tract, while higher doses (up to 3 g/m^2) are not completely absorbed this way and are therefore administered by intravenous infusion. MTX is readily distributed to the body tissues and its renal elimination from the bloodstream follows metabolism by hepatic aldehyde oxidase to 7-hydroxy-MTX and by the intestinal flora to 4-amino-4-deoxy-N-methylpteroic acid [16,17].

The aim of this work is the encapsulation of MTX within SLNs prepared by the coacervation technique.

Coacervation is a well-known technique for nanoparticle preparation, especially for those nanoparticles made of natural polymers, like chitosan [18] and albumin [19]. Phase separation of these polymers as nanoparticles can be obtained either for nonsolvent addiction [19] or ionic complexation with an opposite charged polymer [18]; this step can [19] or cannot [18] be followed by a hardening step. In particular, albumin has been widely used for the delivery of various chemotherapeutic agents, and antineoplastic drugs [20]. In literature, lipid nanoparticles instead have always been prepared with different techniques, such as hot homogenization, microemulsion dilution or cooling [101–103]: coacervation is a new method for lipid nanoparticle preparation, developed by the research group of the authors of this paper [13,14], and is based on fatty acid precipitation as nanoparticles, owing to proton exchange between a soap micellar solution and an acidifying solution.

The in vitro cytotoxicity of drug-carried SLNs was evaluated and compared with that of drug solution: this can be a good perspective in order to reduce therapeutic doses and drug resistance in vivo. In order to verify whether SLNs can improve the enhanced permeability and retention effect, increasing drug plasmatic half-life, drug pharmacokinetics (PK) in male Wistar rats was also studied after intravenous administration of MTX-loaded SLNs and MTX solution. Breast cancer was induced in female Fisher rats through transplantation of the syngenic rat breast cancer cell line, Mat B-III, and biodistribution within neoplastic tissue

was evaluated after intravenous administration of MTX-loaded SLNs and MTX solution. Further investigation was performed on male Wistar rats to verify if encapsulation within a lipid matrix can enhance MTX gut uptake.

Since MTX is a hydrophilic molecule, encapsulation within SLNs was performed through hydrophobic ion pairing: in literature, ion pairs between MTX and surfactants are reported [21], but they are too pH sensitive for the coacervation process. Therefore, new ion pairs were prepared between MTX and counter ions in order to increase drug encapsulation efficiency within nanoparticles.

MATERIALS E METHODS

Materials

Citric acid, phosphoric acid and acetic acid were from Azienda Chimica e Farmaceutica s.p.a (Fiorenzuola d'Arda, Italy), 80% hydrolyzed polyvinyl alcohol (PVA) 9000–10,000 Mw was from Sigma (Dorset, UK); sodium palmitate (Na-P), methotrexate disodium salt (Na_2MTX), potassium permanganate, chlorexidine digluconate (CLO), dequalinium dichloride (DEQ) were from Fluka (Buchs, Switzerland). Sodium acetate and tromethamine were from Merck (Darmstadt, Germany). Ammonium acetate was from Sigma Aldrich (MO, USA). Acetonitrile and methanol were HPLC grade and purchased from Carlo Erba (Milan, Italy). Deionized water was obtained by a MilliQ® system (Millipore, MO, USA). All other chemicals were analytical grade and used without any further purification.

Methods

Ion pair preparation & characterization

Hydrophobic MTX ion pairs were prepared by adding a solution of Na_2MTX (1 mg/ml) to a solution of counter ion (CLO or DEQ) at 1:1 molar ratio. After mixing, the solution was left to settle at 25°C for 1 h and then centrifuged at 55,000 g for 10 min (Allegra™ R64 centrifuge, Beckmann Coulter); the precipitated ion pair was dried overnight under vacuum.

Na_2MTX: counter ions molar ratio was measured on the dried precipitate; this powder was dissolved in a proper volume of benzyl alcohol and the MTX amount was measured after extraction with 0.1 N HCl and HPLC-UV analysis (as described later); the amount of counter ion present in the precipitate was calculated by subtracting the MTX amount recovered by HPLC from the total weight of dried precipitate. HPLC-UV measurement was preferred to simple spectrophotometric detection in order to exclude absorbance interferences by counter ions. MTX quantitative extraction was needed to avoid injection of water insoluble

ion pairs in the hydrophilic mobile phase. Ion pair solubility in water was calculated, determining Na_2MTX in the supernatants by HPLC-UV.

Ion pair apparent partition coefficient (P_{app}) was measured by dissolving a known amount (~2 mg) of Na_2MTX in 2 ml water and shaking for 10 min with an equal volume of benzyl alcohol in a separator funnel, in the absence and in the presence of the counter ions at 1:1 molar ratio; the system was allowed to settle for 1 h, then a sample of the water-phase was centrifuged and Na_2MTX was determined by HPLC-UV. Benzyl alcohol was chosen as the organic phase, since it is a good solvent for H_2MTX, that is, the neutral form of MTX (as described later). The P_{app} was calculated according to the reported formula.

$$P_{app} = \frac{[MTX]_{benzyl\ alcohol}}{[MTX]_{water}}$$

where:

$[MTX]_{benzyl\ alcohol} = [MTX]_{water} - [MTX]_{o\ water}$

$[MTX]_{water}$ = MTX concentration in water after shaking with the organic phase

$[MTX]_{o\ water}$ = initial MTX concentration in water

SLN preparation

Palmitic acid SLNs were prepared according to the coacervation method described in a previous paper [13]. Briefly, Na-P and PVA and counter ions were dispersed in water and heated under stirring (300 rpm) just above the Krafft point of Na-P (50°C) in order to obtain a clear solution. Na_2MTX was dissolved under stirring in the micellar solution and then citric acid solution (coacervating solution) was added drop-wise up to pH 4.5. The obtained suspension was then cooled in a water bath under stirring at 300 rpm until 15°C temperature was reached. The counter ions and the drug were added to the micellar solution separately in order to form the ion-pair in situ. In fact, this was needed because of the poor dissolution rate of the solid ion-pair itself in the micellar solution. Then the pH of the suspension was neutralised prior to in vivo intravenous administration.

SLNs characterization

Particle size and polydispersity of SLN dispersions were determined by the laser light scattering technique (Brookhaven, MS, USA). Measurements were obtained at a 90° angle on the appropriate water-diluted samples.

Differential scanning calorimetry was performed with a PerkinElmer differential calorimeter (CT, USA). Lipid bulk material and SLNs suspensions were placed in conventional aluminum

pans. Experimental conditions were as follows: scan speed 2°C min^{-1}; temperature range 30–80°C.

Drug encapsulation efficiency (EE%) was calculated as the ratio between the amount of drug encapsulated within the lipid matrix and that used to prepare nanoparticles. For the determination of the encapsulated drug, a known amount of SLNs suspension was centrifuged at 55,000 g and the precipitate was dried under vacuum overnight and then dissolved in benzyl alcohol. MTX was extracted with 0.1 N HCl and analyzed by HPLC-UV.

To assess the encapsulation of the ion pairs within SLNs, MTX-release studies were performed by using a test tube assay, as described in literature [22]: benzyl alcohol was used as the organic receiving phase. In total, 10 ml benzyl alcohol was layered onto the surface of 10 ml MTX-loaded SLNs and the system was kept under mild stirring (50 rpm); at scheduled times a small amount of the receiving phase was withdrawn and the supernatant analyzed spectrophotometrically at 380 nm for MTX determination. Ion pairs in the absence of SLNs were used as blanks. The integrity of the lipid matrix in the experiment was checked by differential scanning calorimetry on SLN suspensions before and after release experiments and verifying that no significant changes were induced in SLN melting enthalpies [22].

SLNs in vitro cytotoxicity

Cytotoxicity of Na_2MTX, MTX:DEQ and MTX:DEQ-loaded SLNs was evaluated on the human breast cancer cell line, MCF-7 and the rat breast cancer cell line Mat B-III. Cell lines were obtained from the American Type Culture Collection (ATCC; MD, USA). MCF-7 and Mat B-III cells were grown in RPMI 1640 and McCoy's 5A modified medium (Sigma, Milan, Italy), respectively, supplemented with 10% heat- inactivated fetal calf serum (Sigma) and were cultured at 37°C in a humidified atmosphere of 5% CO_2-air.

Briefly, cells were normalized at 5000 cells/100 µl in 96-well plates (Sigma) and treated after 72 h with 100 µl of culture medium with drugs at 0.01 or 0.1 µg/ml. Viable cell growth was determined by the cell proliferation reagent WST-1 (Roche Applied Science, Penzberg, Germany), according to the manufacturer's instructions at 24, 48 and 72 h. This colorimetric assay for the quantification of cell viability and proliferation is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. The proliferation of the treated cells was calculated as a fraction of the proliferation of the untreated cells (i.e., cells that had received no drug), in the respective experiment. Eight replicate wells were used to determine each data point and three different experiments were performed.

Cytotoxicity of unloaded SLNs was also evaluated on MCF-7, Mat B-III and rat peripheral blood mononuclear cells (PBMCs) at the two concentrations used. Rat PBMCs were isolated and cultured according to Liu et al. [23].

PK after intravenous administration

MTX:DEQ-loaded SLNs (1 mg/ml MTX) and Na2MTX (1 mg/ml MTX) solution in normal saline were administered to adult Wistar male rats (weight = 500 g) through a catheter surgically positioned in the jugular vein at the dose of 2 mg/kg MTX [24]. At scheduled times, blood samples were collected by means of the same catheter. Each experiment was performed on four rats respectively for MTX:DEQ-loaded SLNs and Na2MTX solution.

Blood samples were centrifuged at 17,000 g for 5 min, then 0.25 ml plasma was added to 0.075 ml 10% trichloroacetic acid solution to precipitate plasma proteins and centrifuged at 55,000 g for 1 min. A total of 0.025 ml 5 M acetate buffer (pH 5.0) was added to the supernatant [25].

The precipitate was treated overnight with 0.1 ml 1M NaOH, in order to extract MTX from plasma proteins. 0.1 ml 10% trichloroacetic solution was added to the sample, and the mixture was centrifuged. The pH of the supernatant was regulated by adding 0.15 ml 5 M pH 5.0 acetate buffer.

Both the samples, plasma and plasma proteins, were derivatized prior to HPLC-fluorimeter analysis: plasma concentration was calculated as the sum of data from plasma and plasma protein.

PK analysis

The PK study entailed a typical model-free approach, the noncompartmental analysis, whose goal is to provide an estimation of PK parameters based on the statistical moment theory. PK parameters are determined by a numerical integration procedure, the trapezoidal rule; the only assumption being that the terminal elimination phase of the drug could be described by a monoexponential equation, following first order kinetics. Noncompartmental analysis was performed by Kinetica 2000 4.1.1 (InnaPhase Corp., MA, USA), with the following parameters: C_0 (concentration at time zero), AUC_{tot} (total area under concentration/ time curve), elimination half-life, mean residence time, C_{ltot} (total clearance), V_z (volume of distribution of the late elimination phase), V_{ss} (volume of distribution at steady state).

Statistical analysis

The statistical approach followed in this research is nonparametric. This approach makes no assumptions about PK parameter distribution, unlike the parametric method, where it is assumed to be normal. Descriptive and inferential statistics (Mann–Whitney test) were performed by SPSS 17.0 (SPSS Inc., USA): all data are presented as median values and range.

All reported p-values are two-sided at the conventional 5% significance level, based on its exact distribution (the asymptotic distribution would be incorrect for a very small sample size).

Biodistribution after intravenous administration

Inbred female Fisher 344 rats, weighing 200–220 g were obtained from Charles River Italia (Milan, Italy). The animals were housed in temperature- and humidity-controlled rooms with a 12-h light/dark cycle and were allowed free access to food and water; they were handled according to European guidelines (Directive CEE 86/609) and the experimental protocol was reviewed and approved by the Animal Ethics Committee of the University of Torino, Italy. Before inoculation, Mat B-III cells grown in serum-containing medium were washed with Hank's-balanced buffer and centrifuged at 1500 rpm for 5 min. Cell pellets (1×10^6 cells) were resuspended in 0.5 ml saline and injected using 1 ml insulin syringes into rats' mammary fat pad. Following tumor cell implantation, all animals were examined daily for the development of tumors for up to 10 days. The animals were randomly assigned to two treatment groups, with at least three animals in each group.

MTX:DEQ-loaded SLNs (1 mg/ml MTX) and Na_2MTX (1 mg/ml MTX) solution in normal saline were administered through a catheter surgically positioned in the jugular vein of rats (weight 250 g) at a dose of 2 mg/kg MTX [24]. At scheduled times (30 min and 3 h) the rats were sacrificed by CO_2-induced euthanasia and organs (liver, spleen, kidneys, lungs, heart, brain and breast tumor) were removed surgically. Each experiment was performed on three rats respectively for MTX:DEQ loaded SLNs and Na_2MTX solution.

Organs were homogenized with UltraTurrax® (IKA, Staufen, Germany) for 5 min in water at a tissue concentration of 125 mg/ml, then 0.25 ml of homogenized tissue was added to 0.075 ml 10% TCA solution to precipitate proteins and centrifuged at 55,000 g for 1 min. The supernatant was added of 0.025 ml 5 M pH 5.0 acetate buffer [25].

Oral uptake after duodenal administration

MTX:DEQ-loaded SLNs (1 mg/ml MTX) and Na_2MTX (1 mg/ml MTX) solution in normal saline were administered through a surgically implanted duodenal cannula to adult Wistar male rats (weight 500 g) at the dose of 2 mg/kg MTX. The surgically implanted duodenal cannula was used because we wanted to avoid the variability of gastric emptying after gavage administration. At scheduled times, blood samples were collected by means of a catheter surgically positioned in the jugular vein. Each experiment was performed on three rats respectively for MTX:DEQ-loaded SLNs and Na_2MTX solution. Blood samples were treated as previously described for intravenous administration.

MTX HPLC analysis

HPLC-UV

Experimental conditions were as follows: LC9 pump equipped with SPD10AV UV-visible lamp and C-R5A integrator (Shimadzu, Kyoto, Japan); column: Ultrasphere C18 250 mm × 4.6 mm (Beckmann Coulter); mobile phase: CH_3CN- 0.05M ammonium acetate 7:93; flow: 1 ml/min; λ_{max}: 302 nm; retention time: 15.0 min [26]. The linearity of the calibration graph was demonstrated by the value (0.9993) of R^2 coefficient of the regression equation. The limit of quantitation was 25 μg/ml; the limit of detection was 10 μg/ml.

HPLC-fluorimeter

Samples were derivitized, according to a literature method: MTX was oxidized to the fluorescent 2,4-diaminopteridin-6-carboxilic acid by reacting the sample with 0.025 ml 5% potassium permanganate for 30 min. The reaction was stopped by adding 0.025 ml 3% H_2O_2 and the sample was injected to HPLC [25].

Experimental conditions for HPLC analysis were as follows: LC9 pump equipped with RF-551 fluorimeter (Shimadzu) and C-R5A integrator (Shimadzu); column: Ultrasphere C18 250 mm × 4.6 mm (Beckmann Coulter); mobile phase: 0.1 M tromethamine phosphate buffer; flow: 1 ml/min; λ_{exc}: 275 nm λ_{em}: 444 nm; retention time: 10.0 min [25]. The linearity of the calibration graph was demonstrated by the value (0.9986) of R2 coefficient of the regression equation. The limit of quantitation was 100 ng/ml; the limit of detection was 50 ng/ml.

RESULTS

Ion-pair characterization

Chlorexidine digluconate is a biguanide and DEQ is biquaternary ammonium salt: both have a double positive charge, which can interact with both -COO- groups of Na_2MTX: in fact, at neutral pH, CLO and DEQ can act as a counter ion for Na_2MTX, causing its precipitation from water solution.

Na_2MTX: counter ion molar ratio in the precipitate was 1:1 for both DEQ and CLO and the aqueous solubility of MTX:CLO was 0.15 mM and that of MTX:DEQ was 0.13 mM.

In Table 1 MTX benzyl alcohol/water P_{app} in the presence and in the absence of counter ions are reported. An increase up to 60-fold was noted for MTX:DEQ ion pair, and to 27-folds for MTX:CLO ion pair, indicating that both ion pairs are partitioned mainly in the organic phase, while Na_2MTX is partitioned mainly in water. It should also be noticed that H2MTX, which is the neutral form of MTX, when the two carboxylic groups and the amino group are deionized, has a lower P_{app} than the ion pairs, even if its solubility in benzyl alcohol is higher than that of Na_2MTX.

Table 1. Methotrexate apparent partition coefficient in the absence and presence of counter ions.

MTX	Apparent partition coefficient
Na_2MTX	0.24
H_2MTX	1.40
Na_2MTX:CLO	6.43
Na_2MTX:DEQ	14.22

CLO: Chlorexidine digluconate; DEQ: Dequalinium dichloride; MTX: Methotrexate.

SLN characterization

In Table 2, composition, mean particle size, melting enthalpy (ΔH) and transition temperature (T_{peak}) of blank and MTX-loaded SLNs are shown. Drug EE% within SLNs is also reported in Table 2. Only a small percentage of Na_2MTX is encapsulated in SLNs, owing to the hydrophilicity of the drug; EE% seems to be influenced by the presence of counter ions, as a 14-fold increase was noted with DEQ as a counter ion, which was greater than that obtained with CLO. Even if the neutral form of MTX, H_2MTX, is less hydrophilic than Na_2MTX, its existence is pH dependent. Since the coacervation process is pH dependent, pH shifts that occur during coacervation determine the existence of either unionized or ionized forms of MTX, regardless of the form used in the starting micellar solution. It is worth noting, however, that in the presence of counter ion, the entrapment efficiency of MTX increases significantly, despite pH shifts occurring during the coacervation process. in addition, particle size seems to be related to EE%, as greater percentages of encapsulated drug determined an increase in mean diameters. In differential scanning calorimetry patterns, shown in Figure 1, no relevant differences in melting temperature were noted among the SLNs, while a decrease of melting enthalpy was noted, compared with that of raw palmitic acid.

Table 2. Solid lipid nanoparticle composition, mean diameters, drug encapsulation efficiency, melting enthalpy and transition temperature.

MTX	Blank SLNs	Na_2MTX SLNs	MTX:CLO SLNs	MTX:DEQ SLNs
Na-P	108 mg†	108 mg†	108 mg†	108 mg†
PVA	200 mg	200 mg	200 mg	200 mg
Na_2MTX		5 mg	5 mg	5 mg
CLO			5.6 mg	
DEQ				5.8 mg
1 M citric acid	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Water	10 ml	10 ml	10 ml	10 ml
Mean diameter (nm)	270 ± 25	280 ± 30	289 ± 50	353 ± 60
Polydispersity	0.010	0.015	0.107	0.189
EE%	–	5%	50%	70%
ΔH (J/g)‡	85.3	ND	103.7	107.4
T_{peak} (°C)‡	63.4	ND	63.9	63.6

†*Corresponding to 100 mg PA.*
‡*Raw palmitic acid: ΔH = 192.7 J/g; T_{peak} = 63.6 °C*
CLO: Chlorexidine digluconate; ΔH: Melting enthalpy; DEQ: Dequalinium dichloride; EE%: Drug encapsulation efficiency; MTX: Methotrexate; Na-P: Sodium palmitate; ND: Not determined; PVA: Polyvinyl alcohol; SLN: Solid lipid nanoparticle; T_{peak}: Transition temperature.

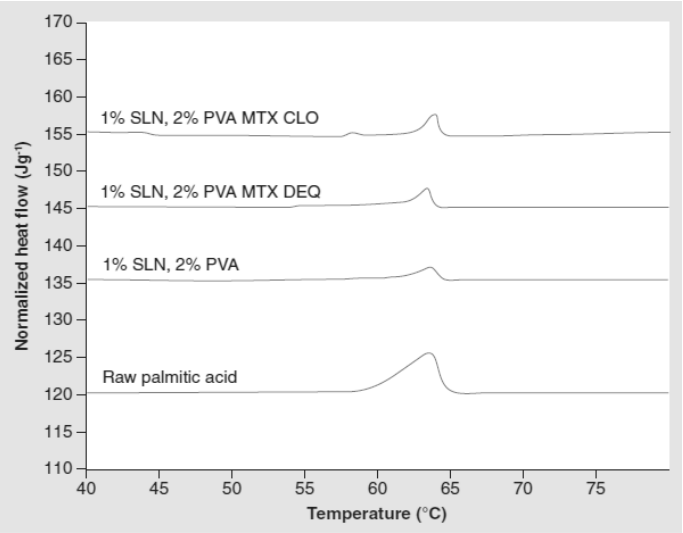

Figure 1. Differential scanning calorimetry patterns of raw palmitic acid, blank and methotrexate ion pairs loaded palmitic acid solid lipid nanoparticles.
CLO: Chlorexidine digluconate; DEQ: Dequalinium dichloride; MTX: Methotrexate; PVA: Polyvinyl acid; SLN: Solid lipid nanoparticle.

To verify the effective encapsulation of ion pairs within SLNs, drug release was studied since the encapsulation of the ion pair in the solid lipid matrix, with its subsequent immobilization, should reduce its release rate [27].

In Figure 2 drug-release profiles from SLN suspensions in the test tube assay are shown: H_2MTX was considered as a blank, instead of Na_2MTX, whose relatively low solubility in the organic phase hindered its use as a reference substance. This method involved two steps: the release of the drug from SLNs to the aqueous outer phase and the migration to the organic phase, where it favorably partitions due to the lipophilicity of the ion pair. Despite the partition step in a nonphysiological medium, this assay is suitable to obtain complete release in a few hours, since the organic phase can easily dissolve a lipophilic molecule or complex. The partition between the SLN suspension and the organic phase is not only influenced by the drug encapsulation within the lipid matrix, but also by the lipophilicity of the drug itself. As the ion pairs are more lipophilic than H_2MTX, their partition towards the organic phase is faster than H_2MTX. On the contrary, when ion pairs are encapsulated within the lipid matrix, drug release to the organic phase is slower than that of both free ion pairs and H_2MTX alone, probably due to the interactions between the lipid matrix and ion pairs. This confirms drug encapsulation within SLNs, which can reduce the drug release rate.

Consequently, the amount of MTX released after 4 h from SLNs is lower than that from free ion pairs and H_2MTX alone. Release experiments were performed in 4 h for comparative purposes, even if further release can be obtained at longer times (data not shown).

For all the subsequent in vivo studies on rats and in vitro studies on cells MTX:DEQ-loaded SLNs have been used instead of MTX:CLO loaded SLNs, since a higher EE% within

nanoparticles is obtained with MTX:DEQ ion pairs: this can be due both to the highest P_{app} and to the lower water solubility of the MTX:DEQ ion pair.

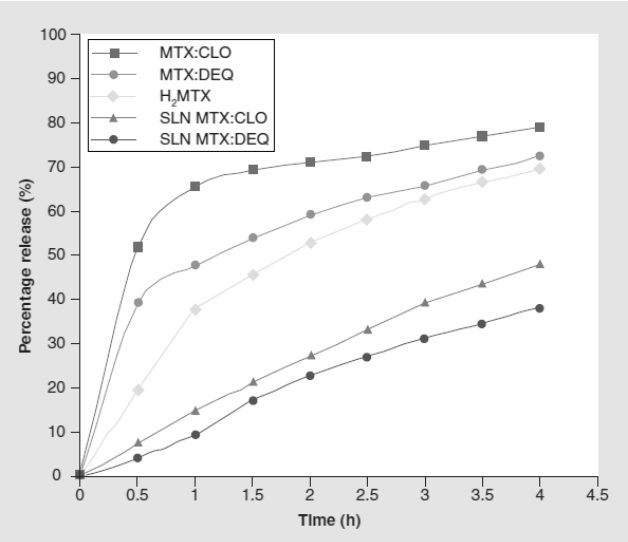

Figure 2. Methotrexate release in benzyl alcohol from free and solid lipid nanoparticle loaded methotrexate ion pairs according to the test tube assay.
CLO: Chlorexidine digluconate; DEQ: Dequalinium dichloride; MTX: Methotrexate; SLN: Solid lipid nanoparticle.

SLNs in vitro cytotoxicity

Figure 3 shows the survival histograms of MCF-7 and Mat B-III cells after 24-, 48- and 72-h exposure to the different MTX formulations under study, Na2MTX, MTX:DEQ and MTX:DEQ-loaded SLNs. In MCF-7 cells, the drug-loaded SLNs showed a significant increase in growth inhibition compared with free MTX and MTX:DEQ at the higher concentration used. Mat B-III cells were more sensitive to MTX:DEQ-loaded SLNs since they showed a higher growth cell inhibition at the lower concentration used. It is worth noting, after 72-h exposure at the higher concentration used, MTX:DEQ-loaded SLNs caused a strong decrease of Mat B-III cell growth to approximately 15% of control cells. Unloaded SLNs were well tolerated, no cytotoxicity of the unloaded SLNs being observed in MCF-7, Mat B-III and PBMCs after 24-, 48- and 72-h exposure at the two concentrations used, as cell viability was not decreased compared with control cells.

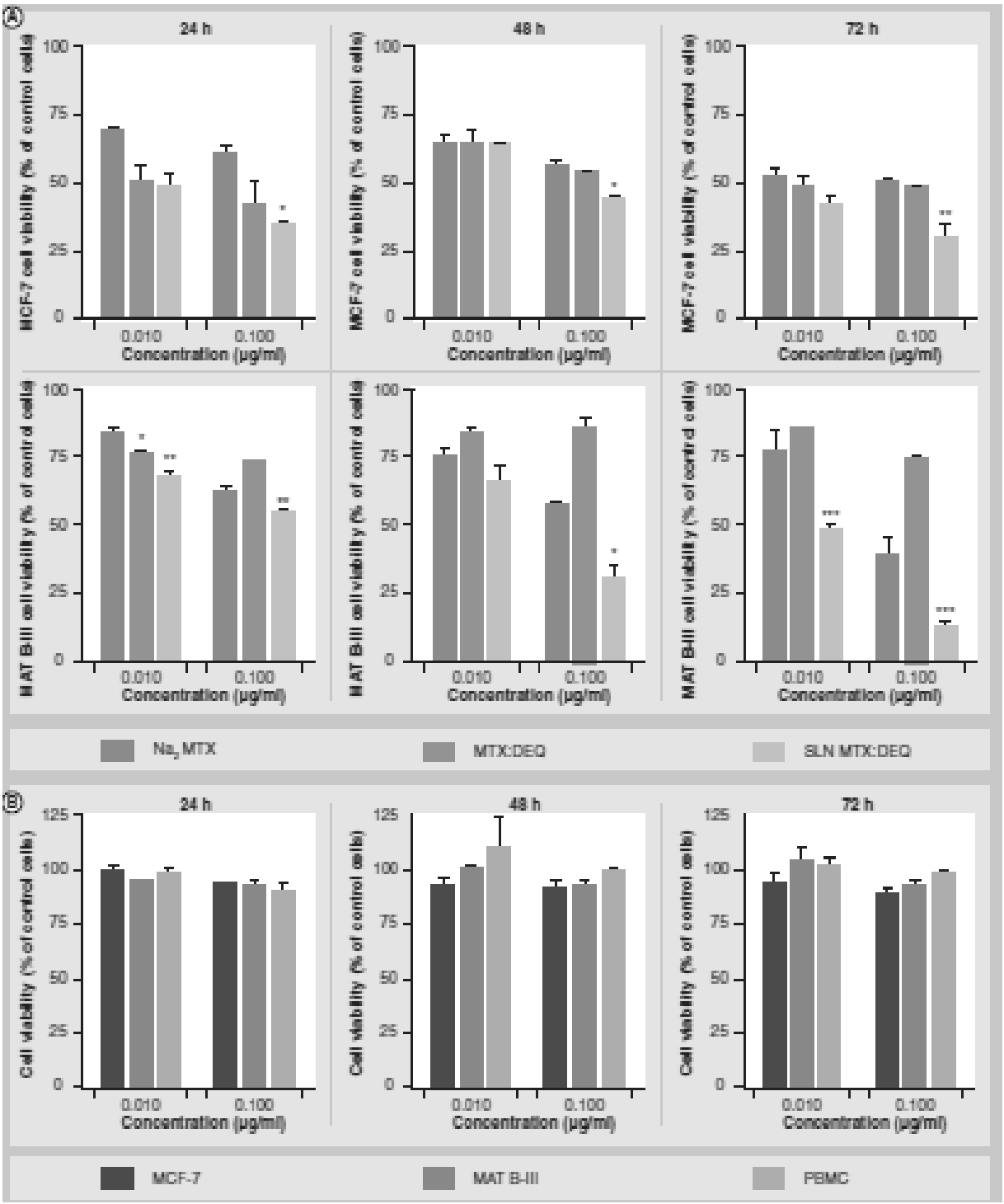

Figure 3. Cell viability after 24-, 48- and 72-h exposure to Na**2**MTX, methotrexate: dequalinium dichloride or methotrexate: dequalinium-loaded solid lipid nanoparticles (0.01 and 0.1 µg/ml MTX) on MCF-7 or Mat B-III cells and to unloaded solid lipid nanoparticles**. (A & B)** Correspond to unloaded SLNs at the same lipid concentration of 0.01 and 0.1 µg/ml MTX:DEQ-loaded SLNs, respectively) on MCF-7, Mat B-III or rat PBMCs. Cell viability is determined as the ratio between treated cell and untreated control cells (100%). Values are means ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (vs free drug). DEQ: Dequalinium dichloride; MTX: Methotrexate; PBMC: Peripheral blood mononuclear cell; SLN: Solid lipid nanoparticle.

PK after intravenous administration

In Figure 4, plasmatic profiles of MTX after intravenous administration of Na_2MTX solution and MTX:DEQ loaded SLNs are shown. As can be noted, encapsulation within SLNs determines an increase in drug plasmatic concentration up to 6 h after intravenous administration, resulting in a significant increase in AUC_{tot}, and consequently mean residence time (Table 3), even if the increase of calculated half-life, instead, is less evident.

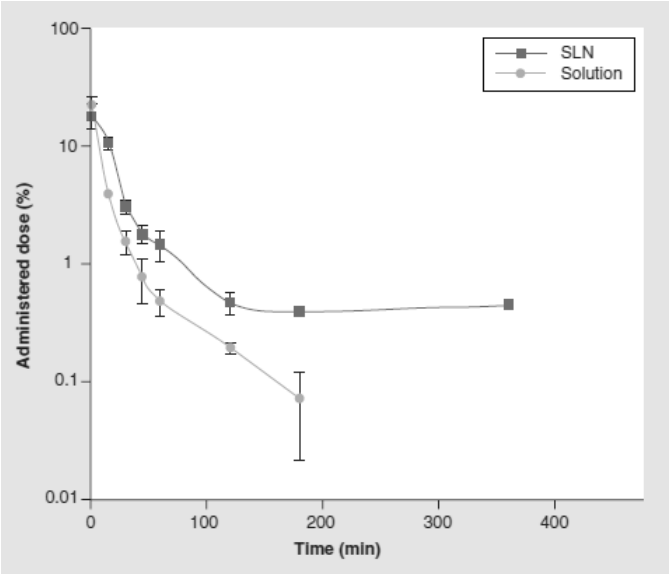

Figure 4. Methotrexate plasmatic profile after intravenous administration through catheterized jugular vein of methotrexate: dequalinium dichloride loaded solid lipid nanoparticles and Na_2MTX solution.
SLN: Solid lipid nanoparticle.

Table 3. Median (range) pharmacokinetic parameters after intravenous administration of methotrexate solution or methotrexate solid lipid nanoparticles.

Pharmacokinetic parameter	SOL	SLNs
Concentration at time zero (mg/l)	5.20 (3.49–5.67)	4.75 (3.85–5.57)
Total area under concentration/time curve (mg*l/min)	70.9 (53.4–72.8)	93.6 (72.9–132.3)*
Half-life (min)	45.3 (38.7–65.0)	53.1 (38.7–87.6)
Mean residence time (min)	28.3 (26.1–31.1)	41.6 (36.5–49.6)*
Total clearance (l/min)	0.010 (0.010–0.013)	0.010 (0.006–0.013)
Volume of distribution of the late elimination phase (l)	0.45 (0.35–0.66)	0.31 (0.24–0.42)
Volume of distribution at steady state (l)	0.19 (0.17–0.28)	0.17 (0.19–0.28)

*$p < 0.05$.
SLN: Solid lipid nanoparticle; SOL: Methotrexate solution.

Biodistribution after intravenous administration

In Figure 5A the biodistribution of MTX after intravenous administration of Na2MTX solution and MTX:DEQ-loaded SLNs is shown. It can be noticed that the drug is distributed mainly in the liver, where it is metabolized, and in the kidneys, which are responsible for elimination. As for breast cancer, after 30 min (Figure 5B), drug concentration in the organ is higher for

Na_2MTX solution than for MTX:DEQ-loaded SLNs. Cancer cells are rapidly dividing cells, where MTX can accumulate easily as a consequence of their requirement for high amounts of folates for replication; when the drug is loaded in the SLN, its availability for cancer cells is lower (even if cytotoxicity towards cancer cells is higher), so that a lower amount is accumulated in breast cancer compared with drug solution.

After 3, 6 and 24 h (Figure 5B), the drug concentration in breast cancer is higher for MTX:DEQ-loaded SLNs than for Na_2MTX solution. This might be due to nanoparticle extravasation: 3 h after intravenous administration of Na_2MTX solution, no more relevant drug concentration is recovered in plasma (Figure 4), and consequently drug biodistribution in tissues is decreased. From 3 h after SLN intravenous administration, drug plasmatic concentration is still significant after SLNs administration, and extravasation of drug-loaded nanoparticles towards breast cancer and enhanced permeability and retention effect are probably favored by leaky vascular architecture in neoplastic tissue.

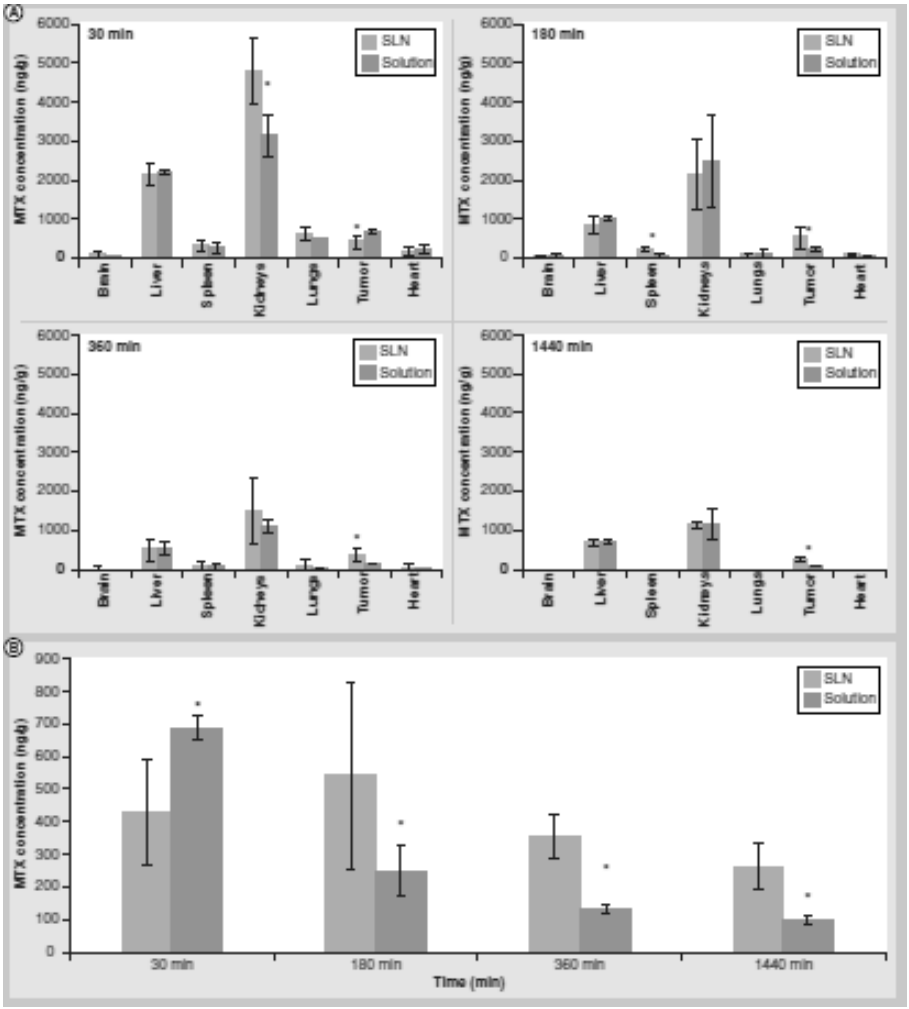

Figure 5. Methotrexate biodistribution after intravenous administration of methotrexate: dequalinium dichloride-loaded solid lipid nanoparticles and Na_2 methotrexate solution**. (A)** Biodistribution in organs at different times after administration. **(B)** MTX concentration in breast cancer after intravenous administration. *p < 0.05. MTX: Methotrexate; SLN: Solid lipid nanoparticle.

Oral uptake after duodenal administration

In Figure 6, the plasmatic profile of MTX after duodenal administration of Na_2MTX solution and MTX:DEQ-loaded SLNs is shown: when drug is loaded in SLNs, drug intestinal uptake, and consequently drug plasmatic concentration is increased.

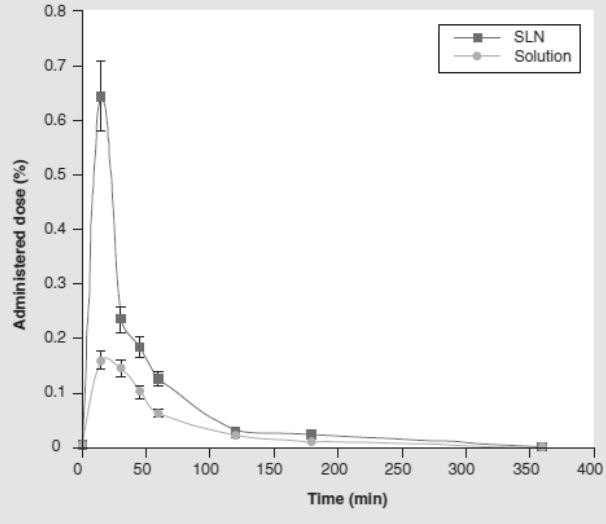

Figure 6. Methotrexate plasmatic profile after duodenal administration of methotrexate:dequalinium dichloride-loaded solid lipid nanoparticles and Na_2 methotrexate solution.
SLN: Solid lipid nanoparticle.

DISCUSSION

In literature, two main examples of MTX-loaded SLNs are present [24,28], but none of them uses ion pairs to increase encapsulation efficacy, while the use of solvents and cosolvents is involved in SLN preparation. In one of these studies, the oral uptake of nanoparticles is considered, but no control with intravenous administration (PK) is done, and no cellular toxicity study is performed on cancer cell lines [28]. In the other study, PK analysis was performed after intravenous administration to rats, the life span of tumor-bearing rats was studied, but no in vitro cellular toxicity and in vivo biodistribution studies were carried out [24]. In this experimental work, SLNs were prepared with a new and solvent-free method and MTX EE% was increased by the use of ion pairs not yet reported in literature; and cytotoxicity of MTX-loaded SLN was tested on a human cell line. Complete PK and biodistribution studies were performed after intravenous injection, also with the use of a rat tumor model; moreover, a preliminary study on MTX-loaded SLNs oral uptake was done.

Methotrexate is a hydrophilic anticancer drug that can be encapsulated in SLNs by using the strategy of hydrophobic ion pairing. CLO and DEQ, two cationic surfactants currently used as antimicrobial agents in mouth wash products, proved to act as counter ions of MTX. Resulting ion pairs had reduced water solubility and increased lipophilicity compared with Na_2MTX. Moreover, both ion pairs markedly increased drug EE% within nanoparticles: the increase was particularly significant for the MTX:DEQ ion pair.

MTX:DEQ-loaded SLNs exhibited an increased cytotoxicity towards human and rat breast cancer lines, MCF-7 and Mat B-III, respectively, compared with Na_2MTX solution (Figure 3). No cytotoxicity of unloaded SLNs, consisting in significant decreases in cell viability, was observed on MCF-7 and Mat B-III cells at the two concentrations used; moreover, no significant cytotoxic effect was observed on rat PBMCs.

Consequently, toxicity cannot be attributed to the vehicle. In the literature [29] increased cytotoxicity of nanoparticle-loaded drugs, compared with plain drugs is reported several times and, even if the precise mechanism of the observed increased cytotoxicity of MTX-loaded SLNs needs more investigation, the obtained result is relevant and suggests in perspective the possibility to reduce the therapeutic dose in vivo.

Drug plasmatic concentration in rats after intravenous administration was increased when the drug was carried by SLNs (Figure 4), as already documented in literature [24]. Compared with literature data, an increase in mean residence time and a decrease in half-life for SLN-loaded drug was noted. The increased persistence of the drug in the bloodstream when SLN-loaded, compared with that occurring after administration of a drug solution, can promote its extravasation in neoplastic tissue, which has a leaky vascular architecture. An increase of drug accumulation in breast cancer is observed at 3, 6 and 24 h after intravenous administration of MTX:DEQ-loaded nanoparticles, probably due to SLN extravasation in the neoplastic tissue; on the other hand, 30 min after administration the free drug uptake by actively replicating neoplastic cells is responsible for major free drug accumulation in breast cancer, compared with MTX:DEQ-loaded SLNs.

In fact, the preferential accumulation of the drug in neoplastic tissue, due to extravasation, is considered in literature the most important rationale for nanoparticle delivery of anticancer drugs [29] and this phenomenon makes this formulation interesting in order to increase the efficacy of MTX treatment. Extravasation is enhanced with nanoparticles whose persistence in the bloodstream is increased by reduced particle size (at least <500 nm), since they are retained by healthy organs (e.g., spleen and liver) to a lesser extent [30]. Moreover, a hydrophilic surface coating that preserves them from phagocytic uptake by the reticuloendothelial system [31]; and in literature it is reported that PVA, which acts asa steric stabilizer for SLN suspensions, confers a hydrophilic surface to colloidal systems [32]. In this study a good drug accumulation in the tumor site was obtained, but an important amount of drug is still recovered in healthy organs. A further reduction of particle size, desirable to improve the tumor selectivity of colloidal systems, can be obtained according to different methods: for instance, extrusion is one of these methods suitable for vesicular systems, such as, liposomes and niosomes, but not for particles with a solid matrix, such as SLNs. Furthermore, particle size in SLNs produced according to the coacervation technique seem to be influenced only by the polymer used as a stabilizer [13,14]: therefore, in the future, an improvement of the selectivity of SLNs biodistribution can be achieved by testing stabilizers other than PVA9000 for SLN preparation, in order to obtain formulations with a greater reduction in particle size.

No PK experiments in tumor-bearing animals were performed, because the first aim was the PK analysis of the new formulation in normal animals and then to evaluate MTX concentration in the tumor. As the size of the implanted tumor is small, the PK is probably not different in these animals. Encapsulation within SLNs also increases drug intestinal uptake after oral administration, even if bioavailability remains much lower when compared with intravenous administration. The increase in plasmatic concentration obtained through SLNs delivery after oral uptake were quantitatively comparable to literature data even with a different T_{max} [28]. In this experiment we evaluated the possibility of administering the new formulation by the gastrointestinal route. The bioavailability of MTX:DEQ-loaded SLNs is higher than that of MTX, but unfortunately the blood concentration is still quite low, probably because of the reduced drug amount absorbed by the gut, and for this reason it is impossible to comment on the PK results after duodenal administration.

Further studies are needed to understand the mechanism of intestinal uptake of SLNs, aimed to optimize strategies to significantly increase SLNs oral uptake.

CONCLUSION

Methotrexate EE% within SLNs was increased by the use of hydrophobic ion pairing, and DEQ was more effective than CLO as a counter ion: therefore, MTX:DEQ-loaded SLNs were tested in vitro and in vivo as drug-delivery systems. MTX:DEQ-loaded SLNs showed an increased cytotoxicity towards human and rat breast cancer cell lines MCF-7 and Mat B-III, respectively, compared with Na_2MTX solution and MTX:DEQ ion-pair, while unloaded SLNs showed no cytotoxicity at the two concentrations used. Moreover, no appreciable cytotoxicity of unloaded SLNs was observed on rat PBMCs. An increased MTX plasmatic concentration and an increased accumulation within breast neoplastic tissue were reached when MTX was loaded in SLNs as ion pairs, compared with MTX plasmatic concentration and tumor accumulation obtained with drug solution, and this makes SLNs promising as a drug delivery system for breast cancer therapy. Drug intestinal uptake after duodenal administration was also increased when MTX was loaded in SLNs, compared with drug solution, but the possible mechanism of this phenomenon is uncertain and needs further research.

FUTURE PERSPECTIVE

In the next few years fatty acid coacervation will be used as a feasible technique to deliver drugs and actives into lipid nanoparticles; their potentiality will be increased with the use of hydrophobic ion pairing of hydrophilic drugs, such as MTX.

Methotrexate-loaded SLNs showed an increased cytotoxicity in vitro towards breast cancer cell lines compared with drug solution, while blank SLNs showed good biocompatibility: further investigations will be performed in the future in order to understand the possible mechanisms underlying the increased cytotoxicity on transformed cells of drug-loaded lipid nanoparticles.

An increased MTX plasmatic concentration and an increased accumulation within breast neoplastic tissue were reached when MTX was loaded in SLNs as an ion pair, compared with drug solution; moreover, the increased cytoxicity in vitro, could suggest a possible dose reduction in vivo. In order to verify an increase of efficacy in vivo, further studies have to be performed. At the moment we are planning further experiments to study the effect of MTX and MTX- loaded SLNs on tumor growth both with histological and biomolecular tests. We will complete the study with the survival rates of the treated animals.

Finally, the possible mechanism of MTX- loaded SLNs intestinal uptake is uncertain and needs further studies in the future.

Financial e competing interests disclosure

The support of the Italian Minister of Research (MIUR) under PRIN 06 program and of Regione Piemonte under Converging Technologies Research Grant is gratefully acknowledged. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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