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In vitro and in vivo methotrexate disposition in alveolar macrophages: Comparison of pharmacokinetic parameters of two formulations

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Abstract

MTX-liposomes, prepared with a polymerised core (LSP), were administered in anaesthetised rats by pulmonary instillation versus free drug. No toxicological effects were macroscopically observed. After each time point: 15, 30, 60 and 90 min, animals were humanely killed and analyses of radio-signal were done. This approach allowed recovery of MTX or breakdown products within biological samples. Previously, kinetics of MTX cellular uptake was performed to identify the cytotoxic concentration of drug formulation for human macrophage. Flow cytometry was set-up to characterise liposomal uptake by ex vivo pulmonary macrophage. Cells were isolated by bronchioloalveolar washes from animals. Results have shown clear different pharmacokinetic parameters between free MTX and the liposomal form of MTX. Unlike classical liposomes, which are mainly taken up by the reticulo-endothelial system, LSP-MTX was not targeted to spleen or kidney. The route of administration could be an explanation of this phenomenon. In addition, LSP-MTX was more retained by the lung tissue. Moreover, free form of the drug reaches easily lymph node. This latest result should be taken into consideration for neoplasic disease and more specifically when lymph nodes are a way for pulmonary metastasis. Finally, LSP-MTX should be tested in physio-pathological model of lung cancer to evaluate the influence of the variation of liposomal formulation pharmacokinetic parameters on the drug efficacy.

Keywords: Methotrexate; Lungs; Pharmacokinetic; Macrophages

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1. Introduction

Since the work of Kimelberg et al. (1976), entrapment of drug of interest into liposomes, such as methotrexate, has been widely used. The main purpose was to improve the drugs therapeutic index by decreasing putative toxic side effect and concomitantly increasing drug activity. Classical liposomes made of phosphatidylcholine and cholesterol is well known to be quickly catabolised by the reticulo-endothelial system, and thus have a very low plasma half-life. Consequently, others polyethylene glycol-based formulations have been developed to circumvent this problem (Moghimi and Szebeni, 2003). Nevertheless, there are pathologies in which the uptake by the reticulo-endothelial system of drug-loaded particles could be of interest. More precisely, for diseases due to macrophages dysfunction, it is possible to use the classical liposome property to quickly reach the specific targeted area leading to reduce toxicity for adjacent tissues. One way to be close to the drug target is a direct local injection (in situ). This approach has been mostly realised in colon carcinoma preclinical model of tumor bearing mice (Ito et al., 2003), and also in clinical pilot studies by endoscopic local injection of anticancer drugs bound to carbon particles. Others compound such as 9-nitro-20(s)-camptothecin have been administered directly into the lung of patients with advanced pulmonary malignancies (Verschraegen et al., 2004). In the following experiments, the administration route and the type of drug carrier are performed to improve methotrexate therapeutic index.

Methotrexate is a folic acid antagonist. Its cytotoxic potency is based on a non-competitive inhibition of an intracellular enzyme: the dihydrofolatereductase. Systemic toxicity of the MTX was mainly observed within kidney (Evans et al., 1986). The aim of these experiments was to determine the liposomes trend to be taken up by pulmonary macrophages, which are present in very high quantities inside the alveolar epithelium. Therefore, these targeted cells could become a transient methotrexate storage compartment, and should release the drug at this level to increase local disposition of methotrexate throughout alveolar area. Obviously, such "sustained release" has to be done with dosage inferior to values inducing cytolysis.

Firstly, fluorescent and radio-labelled liposomes have been incubated in human macrophage cells culture to check the putative toxicity of the formulation. Then, in a second set of experiments, internalisation of liposome by THP-1 cells was analysed by radioassay. Fluorescent liposomes were administered in vivo and fluorescence emission measurement was realised to monitor particle uptake occurrence. Finally, pharmacokinetic studies after pulmonary instillation were performed in comparison with a free drug administration.

2. Materials and methods

2.1. Production of radio-labelled and DiI-labelled liposomes (LSP)

LSP are liposomes that consist in a mixture between polymeric hydrophilic phase (collagen and carrageenan) and a lipophilic phase. The phospholipids used were from Lipoid (Ludwigschafen, Germany), and all other chemicals were from Sigma–Aldrich (St. Quentin Fallavier, France). Preparations were done as previously described (Moutardier et al., 2003). Briefly, 200 mg of phospholipid mixture (PC/PI/C, 2:1:1; namely phosphatidylcholine, phosphatidylinositol and cholesterol) was added to 2 ml of acetone–ethanol 1:1 (v/v), 10 μCi of ethanolic [^{14}C]-dioleoyl-phosphatidylcholine (NEN, Zaventem, Belgium) and DiI (dioctadecyloxacarbocyanine perchlorate 2.5 mM, Molecular Probe, Interchim, France) was added. The solvents were dried under nitrogen. One hundred and twenty milligrams of collagen was added to 26 mg of a 1:1 (w/w) mixture of iota and kappa carrageenans (SBI, L'isle sur Sorgue, France). This mixture, corresponding to polymeric phase, was added to the lipidic film, and then rehydrated with 2.5 ml of NaCl (0.9%, w/v) at 75 °C. The preparation was sonicated and filtered by tangential diafiltration (miniultrasette, Pall-Filtron, St. Germain en Laye, France). Preparation was sterilised by heat with positive pressure on a 22 μm membrane (type rezist, Labo-moderne, Paris, France). Finally, suspension of double-labelled ([^{14}C]-DOPC)/(DiI dioctadecyloxacarbocyanine perchlorate 2.5 mM, Molecular probe) liposomes was obtained.

2.2. Preparation of liposomal methotrexate

The same procedure as above was repeated, but the radio-label was exclusively on the entrapped anticancer

drugs, with 30 μCi of MTX added at the hydration step: [^{3}H]-methotrexate ([^{3}H]-MTX), (NEN, Zaventem, Belgium). The radio-labelled drug, methotrexate was dissolved in the appropriate solvents. The preparation was carried out as above, but with the labelled drug diluted within a solution of cold drug, in order to obtain a liposomal suspension containing a similar amount of drug as might be used in the therapeutic situation. To obtain liposomes size reduction, 2 mM chenodeoxycholate was added during homogenisation. Stabilities and methotrexate release capabilities of liposomal formulations were checked as previously described (Moutardier et al., 2003) and showed distinct potencies between free drug and methotrexate-loaded polymerised liposomal suspension.

2.3. Mean diameters of liposomal formulations

The mean diameters and number of liposomes were determined by quasi-elastic light scattering. The samples were analysed using a Sematech RTG3 analyser (Sematech, Nice, France) with a 5 mW helium/neon laser source giving a laser beam of 632.8 nm.

2.4. Cell lines and MTS cytotoxicity assay

The cell line THP-1 (Tsuchiya et al., 1980) were maintained in 25 cm^2 flasks in RPMI-1640 medium containing 10% foetal calf serum, 1% L-glutamine and in the absence of antibiotics (BioWhittaker, France). Routinely seeding was performed when cells reached 5×10^6 to 10×10^6 ml^{-1}. For cytotoxicity studies, 4×10^5 cells were plated in 96-well plates and incubated with 50 μl of empty liposomes at various concentration, to determine the cytotoxic concentration able to destroy 50% of initial cell amount (CC_{50}) and indication about formulation toxicity for in vitro and in vivo experimentations. Cells were incubated with formulations during 72 h, then dye (MTS, [(3-carboxymethoxy-phenyl)-2(4-sulfophenyl)-2H-tetrazolium], Promega, France) was added and coloration analysed, after 4 h incubation at 570 nm, in a titertek multiscan (Beyneix, France).

2.5. Quantification of LSP-MTX internalisation in THP-1 cells by radio-assay

THP-1 cells were incubated for 120 min with 0.650 μg lipidic phase equivalent of LSP-MTX. A 10^6 THP-1 cells were first incubated with the indicated liposomal formulations for 120 min, washed twice with RPMI-1640 medium containing 10% fetal calf serum, and then samples were performed for analysis (1:50 dilution). Samples were taken in order to determine the radioactivity level inside cells. After washing (three time in the aforementioned medium), clean cells were incubated at 50 °C for 16 h in 1 ml of soluene (Packard, Meridien, USA) before the addition of 10 ml of Picofluor 40 scintillant (Packard, Meridien, USA) and counted for radioactivity.

2.6. Quantification of LSP-MTX internalisation in alveolar macrophages by flow cytometry

Nine male rats (250 g weight) of the Wistar strain (Iffa Credo SA, L'abresle, France) were placed randomly in individual cages. Forty-eight hours before the experiment, they were given a glucose diet (50 g l^{-1}) and 24 h before were put on starvation. The rats received 160 μl of liposome suspension (LSP-MTX corresponding to 6.5 μg of lipidic phase) by intratracheal administration. Liposomes were labelled with DiI as fluorescent probe to allow flow cytometry analysis. Animals were maintained under anaesthesia during experimentation. Rats were sacrificed at each time point and alveolar macrophages were obtained by six bronchioloalveolar washes with 3.5 ml NaCl 0.9% at 4 °C, according to the method of Suntres et al. (1993). Then, cells were purified by centrifugation and washed three times in PBS, counted in Malassey chamber and analyzed with a rhodamine filter (FACSCaliber, Becton-Dickinson).

2.7. Whole body distribution of methotrexate formulations after intratracheal administration

Eight-independent groups of six male Wistar strain rats (320–350 g weight) (Iffa Credo SA, L'abresle, France) were placed in individual cages at random. Forty-eight hours before the experiment they were given a glucose diet (50 g l^{-1}) and 24 h before were put on starvation. Animals were anaesthetised with sodium pentobarbital 0.5 ml kg^{-1} i.p. (Sanofi, France). The rats received 160 μl of liposome suspension by intratracheal instillation, with a eight Fch nasal-gastric probe (Vygon, France), just above the tracheal bifurcation. This dosage corresponds to 4 mg kg^{-1} of MTX free or

in a liposomal form. Liposomes, radio-labelled with [^{3}H] (10 μCi) on MTX or free MTX, were administered at time 0 h. Then, animals groups were sacrificed at 15, 30, 60 and 180 min. Two groups of rats treated by free MTX or liposomal MTX were analyzed at each time point. The whole blood was recovered at three levels: carotid (median cervicotomiae), aorta and cava (median xypho-pubian laparotomiae). The following organs: right kidneys, spleen, liver (right lobe), lungs (inferior right lobe) and mediastinal lymph nodes, as well as bronchioloalveolar washes and urine (total volume excreted), were sampled at each time point. The organs or samples were kept at 4 °C until processing. The whole blood was centrifuged at 4 °C to separate the plasma from the erythrocytes. The organs were washed in isotonic phosphate buffered saline, weighed (100 mg for tissues and 100 μl for fluids) and a sample of each taken and stored at −20 °C. The tissue samples were incubated at 50 °C for 16 h in 1 ml of soluene (Packard, Meridien, USA) before the addition of 10 ml of Picofluor 40 scintillant (Packard, Meridien, USA) and counted for radioactivity.

2.8. Calculations and statistics

AUC (μg min ml^{-1}) corresponds to the area under the kinetics curve of the drug appearance in the compartment, linked to the initial dose at $t = 0$ in the apical medium. AUC $= \int_0^{180} tC_p\,dt$ (C_p is the concentration of the test molecule during the time course of the experiment). AUC values were calculated using the trapezoidal rule. Results are expressed as means ± S.E.M. and analysed by ANOVA for repeated values. The differences were determined by Fisher's test protected least significant difference (PLSD) or the more discriminative Scheffe's test at a confidence level of 95%.

3. Results

3.1. Cytotoxicity and cellular uptake of formulations

The LSP size measured by QELS was 138 ± 42.2 nm ($n = 16$). After 2 h incubation, there was a clear difference between the free drug and the LSP in regards to formulations cytotoxic effect. CC_{50} was 30 μM for entrapped drug and 64 μM for free drug (Fig. 1). Previous works have shown that empty LSP was toxic for cell at high dosage and CC_{50} was 0.54 mg ml^{-1} of incubation medium (data not shown). Quantities of loaded MTX within 0.5 mg ml^{-1} of LSP, correspond to a concentration of 2.2 mM of MTX. While CC_{50} for entrapped drug is smaller than CC_{50} for non-loaded LSP, cytotoxic effect on cells was clearly due to MTX and not the result of any LSP components toxic effect. Comparisons of kinetic uptake between the two formulations have shown that LSP-MTX formulation was more rapidly active than the free drug suspension (Fig. 2). These data were confirmed by analysing experiments performed with radio-labelled MTX. Intracellular levels of drugs are

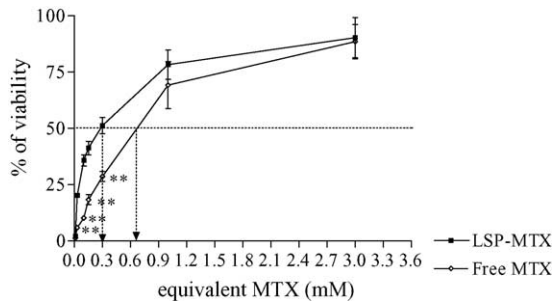

Fig. 1. Comparative cytotoxic effect of LSP vs. free MTX on the human macrophage cell line (THP-1) at 37 °C, shown as the percent of viable cell with respect to methotrexate dosage. Results are expressed as means ± S.E.M. ($n = 3$) in independent experiments. ** Indicates a significant difference between the two formulations at given dosages (t-test, $p < 0.01$).

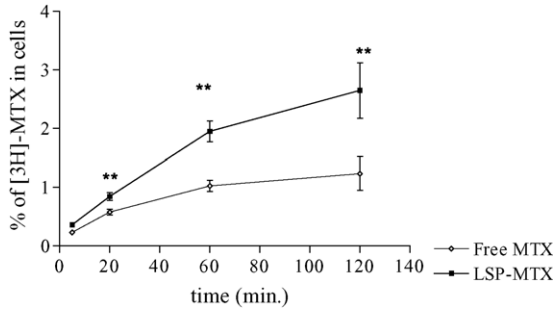

Fig. 2. Comparative cellular uptake of free methotrexate and liposome-loaded methotexate shown as the percent of recovered radio-signal within cells during the time of experiment. Results are expressed as means ± S.E.M. ($n = 4$) in independent experiments. ** Indicates a significant difference between the two formulations at given time point (t-test, $p < 0.01$).

expressed as a percentage of the initial radioactivity added at the beginning of experiment. The amounts of [^{3}H]-MTX and/or breakdown products, found within isolated cells, showed a more pronounced MTX level, when provided by LSP formulation. Liposomal formulation of the drug significantly increased cellular accumulation. Liposomal drug encapsulation increased by two-folds the accumulation of drug into macrophages (Fig. 2).

3.2. Flow cytometry

Previous experiments have demonstrated the in vitro LSP uptake. Then, we have studied the capabilities of liposomal formulation to reach alveolar macrophages after intratracheal administration to anaesthetised animals. In these experiments, LSP-labelled with a lipidic fluorescent probe were used. After intratracheal administration, alveolar macrophages were recovered by bronchioloalveolar washes with 3.5 ml NaCl 0.9%. This ex vivo approach could support the LSP uptake mechanism observed in vitro within alveolar macrophages. As seen in Fig. 3, the mean fluorescence intensity of cells treated with LSP-DiI was 0.6%. The frequency distribution of DiI-positive cells (i.e., numbers of cells plotted as a function of fluorescence level) indicates that the majority of alveolar macrophages express the probe on their surface. To confirm that EDTA/CDCA treatment and rinses removed the free

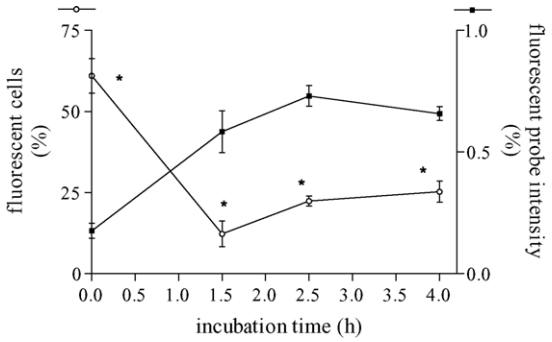

Fig. 3. Liposomal formulations were administered in vivo by the intratracheal way; bronchioloalveolar washes were performed at time 0, 1.5, 2.5 and 4 h in order to isolated alveolar macrophages. Then, quantities of fluorescent dye were analysed to show differential uptake according to time. Results are expressed as means ± S.E.M. ($n = 3$) and are analysed by ANOVA. *Indicates a significant difference between time 0 h vs. time 1.5 h, 2.5 h and 4 h (t-test, $p < 0.05$).

or the non-associated liposomes from the cell surface, the washed supernatant was concentrated and tested for the presence of the DiI. There were no detectable signals.

These data clearly showed that liposomes are able to penetrate inside the cells, or to be coated at the surface of the alveolar macrophages. However, it cannot be excluded that these signals such as fluorescent probe or radioactive marker could correspond to LSP breakdown products because these are not covalently linked to the liposomes.

3.3. Body distribution of the LSP after intratracheal administration

First, histological examinations of epithelium after treatment showed no direct effect of the drug on lung tissue, especially lesions of endothelial tissue (data not shown). In order to study the bioavailabilities of the two MTX formulations, tissues samples or liquid were collected during the procedure (Table 1). Analysis of the radioactivity within bronchioloalveolar liquid revealed more important signal in samples obtained from animals treated with free drug after 30, 60 or 180 min. There was no difference between the two experimental groups at the beginning of experiment. From 15 to 180 min, a marked decrease has occurred in the free MTX group as well as in the LPS-MTX group. This fact mimics the passage from lung alveoli to the pulmonary tissue or cellular compartment such as macrophage as observed previously. Simultaneously, drugs levels in lungs are expressed as a percentage of the initial radioactivity added. From 30 min, the accumulation in tissue was always higher in liposome group than with free drug. Liposomal MTX encapsulation reduced the level of drug accumulated in bronchioloalveolar washes, while encapsulation of MTX significantly increased its accumulation in lungs. Nevertheless, it cannot be concluded that liposomes-loaded with MTX are more taken up by lungs, since it is possible that these formulations reached compartment such as macrophages or lung surfactant/epithelium and progress throughout the lung tissue.

Radio-labelled MTX or radio-labelled breakdown products of MTX recovered from blood, showed the transport of drug across the pulmonary epithelium. First of all, there is a clear difference within MTX repartition between red blood cells and plasma 15 min after

Table 1
Whole body time distribution of MTX formulations (expressed as a percentage of the initial radioactivity administered at $t = 0$ per g or ml)

Compartment	Time (min)			
	15	30	60	180
Bronchioloalveolar fluid (wash)				
Free MTX	97.2 ± 12.4	70.4 ± 6.2*	74.2 ± 10***	81.5 ± 10.3***
LSP-MTX	75.1 ± 8.1	55.5 ± 3.7	40 ± 4.4	32 ± 3.4
Lungs				
Free MTX	43.2 ± 6.5	13.6 ± 2.3	15.3 ± 3.2	8.6 ± 1.3
LSP-MTX	39.4 ± 5.9	45.7 ± 1.7***	36.1 ± 7.4*	34.5 ± 2.6***
Urine				
Free MTX	5.2 ± 0.4	23.2 ± 5.4	43.2 ± 5.8	51.2 ± 7.3
LSP-MTX	5.3 ± 0.3	27.3 ± 6.1	35.3 ± 6.8	58.3 ± 9.9
Spleen				
Free MTX	6.5 ± 0.4	10.1 ± 1.3	14.5 ± 1.3	22.6 ± 3.2*
LSP-MTX	7.1 ± 0.3	12.3 ± 2.3	14.2 ± 2.4	13.1 ± 1
Liver				
Free MTX	8.9 ± 1.9	7.5 ± 2.8	14.8 ± 3.1	21.8 ± 0.7*
LSP-MTX	11.9 ± 3.6	15.3 ± 0.9*	17.6 ± 2.9	16.4 ± 1.7
Med. lymph node				
Free MTX	16.7 ± 0.7	15.8 ± 0.3	36.5 ± 4.1**	49.5 ± 6.1***
LSP-MTX	16.9 ± 1.1	20.9 ± 0.4	22.3 ± 2.6	24.6 ± 5.2
Venous erythrocytes (vena cava)				
Free MTX	35.4 ± 2.1	35.1 ± 5.1	31.2 ± 2.8	33.4 ± 3.3
LSP-MTX	31.2 ± 4	51.2 ± 6.1**	52.1 ± 4.9**	55.1 ± 4.7**
Arterial erythrocytes (carotid)				
Free MTX	42.9 ± 5.1	31.2 ± 6.3	29.9 ± 7.1	31.1 ± 3.2
LSP-MTX	46.6 ± 2.8	51 ± 4.5*	53.1 ± 4.2*	54.8 ± 5.1**
Arterial erythrocytes (aorta)				
Free MTX	43.2 ± 4.2	34.6 ± 2.7	32.8 ± 5.1	30.7 ± 3.4
LSP-MTX	48.4 ± 1.6	50.2 ± 4.4**	52.3 ± 4.5**	52.8 ± 6.1**
Arterial plasma (aorta)				
Free MTX	5.2 ± 0.8	6.1 ± 1.1	14.8 ± 1.9**	14.9 ± 1.2**
LSP-MTX	4.8 ± 0.5	6.8 ± 1.3	8.7 ± 1.9	8.2 ± 1
Arterial plasma (carotid)				
Free MTX	4.2 ± 0.7	5.8 ± 1.3	15.6 ± 2.9**	14.8 ± 1.1**
LSP-MTX	5.1 ± 0.3	7.2 ± 1.4	9.4 ± 1.7	8.4 ± 1.4
Venous plasma (inferior vena cava)				
Free MTX	5.2 ± 0.7	6.9 ± 1.2	14.8 ± 1.1**	15.2 ± 3**
LSP-MTX	6.1 ± 0.5	6.5 ± 0.9	9.4 ± 1.8	8.8 ± 2.4
Kidney				
Free MTX	8.2 ± 0.7	18.5 ± 0.9*	15.7 ± 3.1	20.3 ± 1.4*
LSP-MTX	9.3 ± 1.3	13.1 ± 1.8	14.8 ± 5.3	15.8 ± 0.6*

Values are expressed as mean + S.E.M. ($n = 6$) for each experimental group. Differences are analysed by ANOVA and are significant at 95% MTX vs. LSP-MTX. Med: mediastinal.

* Fisher test.

** Scheffe test.

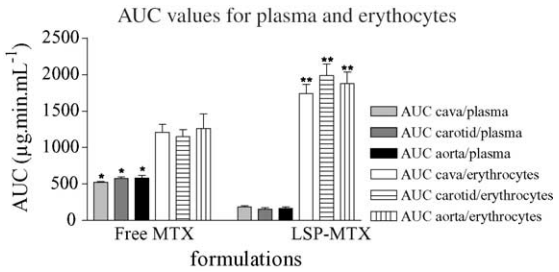

Fig. 4. Whole blood distribution of free methotrexate vs. liposomal form of methotrexate after pulmonary administration to rats. Results are expressed as means ± S.E.M. ($n = 6$) and are analysed by ANOVA. Significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and free MTX vs. LPS-MTX.

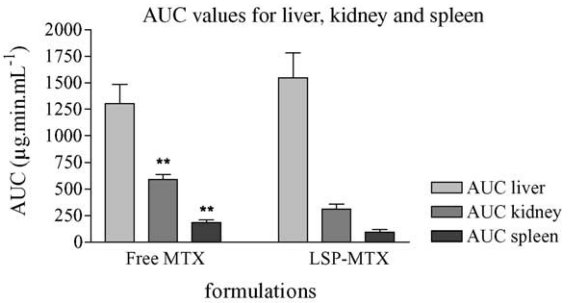

Fig. 5. Liver, kidney and spleen distribution of free methotrexate vs. liposomal form of methotrexate after pulmonary administration to rats. Results are expressed as means ± S.E.M. ($n = 6$) are analysed by ANOVA. Significant at $^{**}p < 0.01$ and free MTX vs. LPS-MTX.

pulmonary administration, radio-signal were higher in red blood cell than in plasma. Next, there are no statistical differences in red blood cell drug level in carotid, aorta, or vena cava during 180 min. There were no variations linked to the time or the localisation of sampling. Before or after the lung, there were no clear variations in MTX level in the blood network whatever the formulation. With this "in vivo multisampling method," it was impossible to differentiate MTX level in arterial or venous network. Analysis of MTX repartition in the blood during experiment with respect to area under the curves (AUCs) provides a global view about the drug behaviour, according to the type of formulation (Fig. 4). When MTX was loaded within liposomes, it was more recovered in red blood cells than in plasma. On the other hand, free MTX was more present in plasma than the drug liposomal form.

In the spleen, free MTX levels were more important than LPS-MTX after 180 min of pulmonary administration. In contrast, MTX repartition in liver has appeared more complex. The signal from the liposomal form was two-fold superior to the free form after 30 min. Although the phenomenon was reversed at the end of the experiment. One hour after administration, the influence of the formulation on the MTX accumulation in lymph node was clearly shown. The signal from the free form of the drug was higher than those obtained with a MTX liposomal formulation. Accumulation of drug or their catabolites in kidney was visible at 30 min and reached a plateau after 180 min. From the 30 min time point, the free form of MTX led to a higher MTX level than the liposomal form that was confirmed by AUCs comparison (Fig. 5). In the spleen, the accumulation was higher with the free MTX group than with the liposome group. Liposomal encapsulation of MTX reduced the tissue accumulation. Liposomal encapsulation of MTX significantly increased liver tissue accretion at 30 min. At this time point the lowest tissue uptake was seen with the free form of MTX. In contrast, liver uptake of free MTX at 180 min was higher than the liposomal form. Finally, comparison of AUC values obtained for the liver compartment, were the same whatever the formulation considered (Fig. 5).

Global pharmacokinetic parameter analysis such as clearance provided important information about the liposomal formulation performance. As shown in Table 2, the non-renal clearance was superior for the

Table 2
Clearances and MTX excretion in urine

Formulations	Renal clearance (ml min^{-1} kg^{-1})	Non-renal clearance (ml min^{-1} kg^{-1})	Total excretion (µg kg^{-1})
MTX	0.10 ± 0.02	0.75 ± 0.13**	51.19 ± 10.45
LSP-MTX	0.33 ± 0.17	1.71 ± 0.34	58.02 ± 20.36

Values are expressed as mean ± S.E.M. ($n = 6$) for each experimental group. Differences are analysed by ANOVA and are significant at 95% MTX vs. LSP-MTX.
** Scheffe test.

liposomal form than the free form of the drug even if the same quantity of metabolite was found in urine. Consequently, the catabolic profile of the drug could be modified by the liposomal formulation, which appears to prevent a rapid uptake by the spleen and/or the kidney.

4. Discussion

Tumor bearing mice is widely used in drug discovery for in vivo cancer studies. However, it is very difficult to obtain plasma sampling due to the size of animals. For this reason, larger animal such as rats have been chosen as a model for this preliminary pharmacokinetic study. Obviously, the next step should be the evaluation of anticancerous compounds efficacy in several formulations. These experiments would allow the evaluation of liposomal or free compound activity in pathological preclinical model such as xenografted athymic nude rat (Rnu/Rnu). The aim of this work was to monitor the behaviour of liposomal formulation versus free MTX after a pulmonary administration, and to evaluate the MTX formulations effect and preliminary tolerance, with a local administration.

First of all, the methods of MTX assay used in the aforesaid work cannot clearly establish if the radio-signal measured in biological samples came from MTX or MTX breakdown products. Although it is very difficult to circumvent this issue, all other approaches such as mass spectrometry coupled to liquid chromatography or covalently linked fluorescent dye to the molecule, exhibit also various disadvantages, and thus all technical tools have equal interest.

Pharmacokinetics results showed that liposomes allowed keeping MTX within the lung. Free MTX plasmatic and mediastinal lymphatic concentrations rose rapidly. This molecule could be then acutely administered through the lung for a systemic and/or lymphatic effect in rats. Others techniques can also be used to check the local concentration of drug in a tissue such as microdialysis. For example, measurement of interstitial concentrations in lung can be monitored following i.v. bolus doses (De La Pena et al., 2001). LSP-MTX also seemed to cross through the lung barrier, but to a lesser extend than the free form. This liposomal form of MTX can have local pulmonary action, but also delayed systemic effects. However, pharmacodistribution studies suggested that pulmonary administration of LSP-MTX would have fewer side effects on systemic tissues (liver, spleen, kidney), although this hypothesis needs to be confirmed. On the other hand, liposomal formulation should not avoid the rapid formation of 7-hydroxy methotrexate, which is the active metabolite of MTX. Lung retention of formulations might change systemic exposure to the drug. This phenomenon was proven in other experiments using intratracheal administration route. Paradoxically greater systemic exposure of the drug had occurred, after intratracheal administration, than after systemic administration (Bleyer et al., 1997).

In previous work, we have shown that the structure of the LSP is different to classical liposomes, because of a polymeric network presence near or in the liposomes (Moutardier et al., 2003). The release rate of encapsulated substances shows the potential of this vehicle to limit the leakage and cytotoxic effects of entrapped molecules. In addition, this particular structure could explain the differences between the two MTX formulations in terms of excretion and targeted tissue. One important point is the capability of MTX to be loaded into red blood cells. Liposomal entrapment of MTX enhanced this loading process. Accordingly, erythrocytes could be promising physiological drug carriers, be an excellent candidate for enhanced macrophage uptake and be useful for targeting any tumor of the reticuloendothelial system. Many studies have been conducted to optimize the potential of red blood cells to behave as drug carrier (Mishra and Jain, 2000). Other physiological carriers such as lipoproteins are of interest to increase the targeting efficiency of active compounds (Clerc et al., 1993). New galenic forms like nanoparticles, polymeric macromolecules or dendrimers are technological tools that can allow and enhance an uptake by secondary physiological carriers. Drugs could be covalently attached onto the carrier surface and provide targeting, imaging and intracellular drug delivery capabilities (Quintana et al., 2002). Otherwise active compounds could be simply loaded inside or around particles (Khopade et al., 2002). The final goal is to improve the drug specificity with the assistance of a physiological carrier.

It is well established that the liver is a clear catabolic area for MTX. Previous works performed in

chronically treated rats had shown that, whatever route of administration, subcutaneous or intraperitoneal, the liver is the main site of MTX worst side effects (Balis et al., 1986). In the present study, pulmonary administration of free MTX or liposomal form of MTX led to a clear differential repartition. Liposomal MTX was less present in the kidney and spleen in term of AUC, but levels within the liver were identical to the free drug. Therefore, a liposomal formulation is expected to decrease the toxic impact of MTX treatment in reticulo-endothelial system, even though this needs further investigations and confirmation with respect to physio-pathological conditions. Nevertheless, free MTX is more present inside mediastinal lymph nodes, which are a part of reticulo-endothelial system, than liposomal formulation. This represents a disadvantage since lymph nodes are the primary way taken by metastasis to increase invasivity of primary tumors. On the other hand, this type of drug distribution could bypass these compartments without being toxic. With other types of liposomes, it is possible to obtain a different reticulo-endothelial system repartition of drug formulation. This biodistribution is liposome composition dependent as shown by Park et al. (1994).

Analyses of clearance provide information about elimination of these MTX formulations. MTX non-renal clearance was superior when the drug was administered as liposomes. A biliary elimination of LSP-MTX could be presumed due to possible biliary tropism cause by a LSP component such as the tensioactive or the lipidic part of liposomes. In rats, Kim et al. (1995) have determined MTX pharmacokinetic and distribution after intravenous or intraperitoneal administration of free and liposomal form of MTX. These data reported that after intravenous administration (equivalent to 4 mg kg^{-1} of MTX), plasma concentrations of MTX; AUC, half-life and mean residence time were significantly increased in the liposome group. In addition, renal and non-renal clearances of MTX decreased significantly. This was probably due to tissue uptake of amount of liposome-MTX and a slow release of MTX. These data confirmed the differences of in vivo behaviour for a drug when loaded into liposomes. Such differences were also observed in our rat model. Actually, the main question raised, “were there any evidence of therapeutic improvement when drug-loaded liposomes modifies clearance or drug targeting?” Further experimentations in pathological model of cancer are needed, but it is likely that a local delivery of anticancerous agent should be appealing and more specific when considering the treatment of lung tumors with a local slow drug release. In this way, LSP formulation could be of interest. This approach was used many years ago by Mc Cullough and Juliano (1979) with a liposomal form of cytosine arabinoside that was administered throughout the lung. The results showed an increase in lung rentention of the liposomal formulation versus the free drug. Obviously, industrialisation and pharmaceutical development problems have delayed launching of such products. Currently, because of analytical tools progress and individualisation of treatment, liposomes approaches seem to be again a putative therapeutic option.

The lung repartition of the aerosolised droplets represents a major parameter to be taken into account when administering drugs by the pulmonary way. The results from Zeng et al. (2000), illustrated that tracheal instillation of liposomes allowed their diffusion in large and small airways, with a uniform distribution in the lung lobes. It has also been reported that distribution of liposomes after tracheal instillation in rats is different among the lung segments: the right inferior lobe received less substance and the right superior lobe accumulated more material. Distribution can be altered by many factors including the animal posture during instillation, the particle size, the volume administered and the ventilation mode (Brain et al., 1976). In our study, we did not look at pulmonary diffusion of the free MTX and the MTX liposomal form. It is possible that some liposomes instilled got trapped in the upper airways and have been eliminated by mucus before having any action. Aerosols infusion appears to be a better method with a more homogeneous distribution within the lung. Index variations reported are then always lower than two-folds (Jurima-Romet and Shek, 1991).

5. Conclusion

Even if these data provide insights about the dynamic disposition of drugs in different formulations, the results of pharmacokinetic studies when animals are maintained under anaesthesia must be interpreted with caution. Currently, in vivo blood sampling in three areas of the vascular network cannot be done by another procedure.

Pharmacokinetics results showed that LSP allowed keeping MTX in the lung. Free MTX plasmatic and mediastinal lymphatic concentrations rose rapidly. This molecule could be then acutely administered through the lung for a systemic and/or lymphatic effect in rats. LSP-MTX also seemed to be passing throughout the lung barrier, but in a less important way. This form could have a really pulmonary action, but also systemic effects. However, pharmacodistribution studies suggested that pulmonary administration of LSP-MTX would have fewer side effects on the reticuloendothelial system, particularly for spleen and kidney.

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