COMPARATIVE EVALUATION OF METHOTREXATE TOXICITY AS SOLUTION FOR INJECTION AND LIPOSOMES FOLLOWING A SHORT-TERM TREATMENT IN A MURINE MODEL OF ARTHRITIS. NOTE I. HAEMATOLOGICAL AND BIOCHEMICAL EVALUATION

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Abstract

The aim of this study was the evaluation of the toxicity of methotrexate (MTX)-loaded liposomes, comparatively with MTX injectable solution, following a short-term treatment in a rat model of arthritis (Freund's adjuvant induced).

We prepared liposomes with MTX itself ("hydrophobic MTX") and with its disodium salt ("hydrosoluble MTX"). The intracellular transport of liposomes has been evaluated *in vitro* on RAW267.4 tumour murine macrophages by fluorescence microscopy using DilC18(3) as fluorescent probe.

Three different doses of MTX preparations (0.2 mg/kg b.w., 0.3 mg/kg b.w. and 0.4 mg/kg b.w.) have been intravenously (i.v.) administered once a week for three weeks. The influence of MTX treatment was evaluated on the haematological parameters and on some biochemical parameters (relevant for the liver and kidney function).

Moderate decrease of haemoglobin and hematocrit was observed 14 days after the last MTX dose in the rats treated with low and intermediate doses of MTX –injectable solution and hydrosoluble MTX-loaded liposomes doses. The leukocyte formula was impaired, predominantly 7 days after the last MTX dose; an increase of the granulocyte count associated with a decrease of the monocyte count were noticed, indicating an equilibrating mechanism for the phagocytes. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were low and irregularly affected, with a moderate increase of the AST values following high MTX doses. Other serum biochemical parameters (glucose, bilirubin, creatinine and urea levels) showed no significant differences compared with the control group.

The results of the study suggest that MTX-loaded liposomes treatment has a reduced toxicity, comparatively with MTX injectable solution treatment in a murine model of arthritis.

Rezumat

Scopul studiului a fost evaluarea toxicităţii lipozomilor cu metotrexat (MTX), comparativ cu soluţia injectabilă de MTX, într-un model de artrită la şobolan (indusă cu adjuvant Freund).

Am preparat lipozomi cu MTX ca atare ("MTX hidrofob") şi cu sarea sa disodică ("MTX hidrosolubil"). Transportul intracelular al lipozomilor a fost evaluat *in vitro*, pe macrofage tumorale murine RAW267.4, prin microscopie de fluorescenţă, folosind ca sondă DilC18(3). Preparatele cu MTX au fost administrate intravenos (i.v.), în trei doze diferite (0,2 mg/kgcorp, 0,3 mg/kgcorp şi 0,4 mg/kgcorp), o dată pe săptămână, timp de trei săptămâni. A fost evaluată influenţa tratamentului cu MTX asupra parametrilor hematologici şi asupra unor parametri biochimici (relevanţi pentru funcţia renală şi hepatică).

Scăderi moderate ale hemoglobinei şi hematocritului s-au înregistrat la 14 zile după ultima doză de metotrexat administrată, la loturile tratate cu dozele mici şi intermediare de soluţie injectabilă de MTX şi lipozomi cu MTX hidrosolubil. Au fost induse perturbări ale formulei leucocitare, cu precădere la 7 zile de la ultima doză de MTX. S-a observat creşterea proporţiei de granulocite odată cu scăderea numărului de monocite, indicând un mecanism de echilibrare numerică la nivel de fagocite.

Activitatea aspartataminotransferazei (AST) şi alaninaminotransferazei (ALT) a fost slab şi neuniform afectată, înregistrându-se uşoare creşteri (îndeosebi ale AST) la dozele mari de MTX. Alţi parametri biochimici (glicemia, bilirubina creatinina şi ureea serică) nu au avut modificări semnificative comparativ cu lotul martor. Rezultatele studiului sugerează că tratamentul cu lipozomi cu MTX prezintă toxicitate redusă, comparativ cu tratamentul cu soluţie injectabilă de MTX, în modelul murin de artrită.

Keywords: methotrexate toxicity, liposomes, adjuvant Freund induced arthritis

Introduction

Methotrexate, an antimetabolite of folic acid, is an antineoplastic agent, which proved to be effective in various neoplastic diseases. It possesses immunosuppressant properties as well. Methotrexate (MTX) is used at high doses in several neoplastic diseases (acute lymphoblastic leukemia, osteosarcoma, etc.), but also, at low doses, in certain autoimmune diseases [3]. With the demonstration, in 1985, that low, intermittent doses of MTX are effective in treating of the patients with rheumatoid arthritis (RA) [5], the range of MTX therapeutic indications has expanded. The high efficacy/toxicity ratio gives the current position of MTX in rheumatoid arthritis therapy as the most effective and well-tolerated disease-modifying antirheumatic drug (DMARD) prescribed to patients, worldwide [8]. MTX is also indicated to the patients with severe forms of psoriasis, not responding to other forms of treatment.

Liposomes have been extensively investigated as drug delivery systems in the treatment of RA increasing the therapeutic index of

antirheumatic drugs. However, high doses may pose the risk of systemic adverse reactions [10].

It has been shown that prolonged circulation by liposomal incorporation increases the therapeutic efficacy of drugs in many cases. Thus, after i.v. injection in rats, pharmacokinetics, including distribution of MTX were significantly modified by the incorporation in liposomes and by the modulation of the liposomal composition while renal and hepatic drug accumulation was significantly reduced [7].

In RA, it has been found that intra-articular administration of anti-inflammatory drugs encapsulated in liposomes results in the increasing of the retention time of the drug in the joints and thus reduce inflammation [9, 11]. In addition, longer retention of the drug in this area may reduce systemic side effects [6]. Also, the arthritic inflammatory process induced *in vivo* (with collagen or Freund adjuvant) was significantly reduced in rats injected i.v. for 4 days with liposomal preparations containing MTX.

The present was aimed to the evaluation of the toxicity of MTX-loaded liposomes, comparatively with MTX injectable solution, following a short-term treatment (21 days) in a rat model of arthritis (Freund's adjuvant induced).

Materials and Methods

The liposomes containing MTX were prepared by two methods: lipid film hydration method and reverse phase evaporation method and were previously analyzed for the size dispersion and MTX entrapment [1,2].

Since the studies on the interaction between liposomes and cells are of particular importance to the design of liposomes acting as high efficiency vectors able to carry drugs to cells, the internalization of various liposomes types by the murine tumour macrophage cells has been investigated. Three types of liposomes have been prepared: conventional liposomes, containing phosphatidycholine (PC), and sterically stabilized liposomes, containing: phosphatidylcholine:cholesterol:polyethylene glycol 2000 – phosphatidylethanolamine (PC:CH:PEG2000-PE) and phosphatidylcholine: polyglycerol poly-12 hydroxystearic acid ester (PC:PGPH). The internalization of MTX free liposomes by RAW267.4 tumour murine macrophages was visualized by fluorescence microscopy using DilC18(3) (1,1′ - dioctadecyl - 3,3,3′,3′ - tetramethylindocarbocyanine perchlorate) (Molecular Probes) as fluorescent probe. RAW 264.7 macrophages were incubated for 2 hours at 37ºC, with Dil labeled liposomes.

Adjuvant arthritis was induced by injecting of complete Freund's adjuvant (CFA, heat killed and dried *Mycobacterium tuberculosis* in mineral oil) to the subplantar region of the left hind paw.

The animals used in the experiment were male Wistar rats, 12 weeks old, brought from an authorized breeding farm. The animals were housed for 7 days in the new environment with a 12-hr light/dark cycle and free access to special rat food provided twice a day (at 8.00 in the morning and again at 17.00 in the evening) and water *ad libitum* all day long. The temperature was maintained between 21-24ºC, while the humidity oscillated between 45-60%. All researches were conducted in accordance with The European Directive 86/609/EEC/24.11.1986 and The Romanian Government Ordinance 37/30.01.2002 regarding the protection of animals used for experimental and other scientific purposes.

After induction of arthritis, 21 days after injection of CFA, the animals were assigned to 10 groups of five animals, which received the following treatments: groups 1;2;3 – hydro-soluble MTX -loaded liposomes, at a dose of 0.2;0.3;0.4 mg/kg b.w.; groups 4;5;6 – hydrophobic MTX -loaded liposomes, at a dose of 0.2;0.3;0.4mg/kg b.w.; groups 7;8;10 – MTX solution for injection, at a dose of 0.2;0.3;0.4 mg/kg b.w.; group 9 – control group, treatment with liposomes, 0.1 mL/100g/kg b.w. The doses were administrated intravenously once a week for 21 days.

The hemogram with the leukocyte formula, serum AST and ALT activities, serum glucose, bilirubin, creatinine and urea levels were evaluated 7 days and 14 days after the last MTX dose. Biochemistry investigations were achieved with the Beckman Coulter analyser using Beckman Synchron LX® kits. The complete blood cell count was achieved with the CELL-DYN 1700 (Abbott Diagnostics) automatic system.

Results and Discussion

In vitro evaluation of the intracellular transport of liposomes

Having in view the importance of liposomes uptake by macrophages for the removal from the blood circulation after intravenous injection, we studied the uptake and quantification of liposomes internalization by the tumour murine macrophages RAW267.4. Images obtained using a epifluorescence microscope (excitation at 530 nm and emission at 580 nm) revealed punctuate intracellular fluorescence, which indicates that the liposomes are internalized (Figure 1).

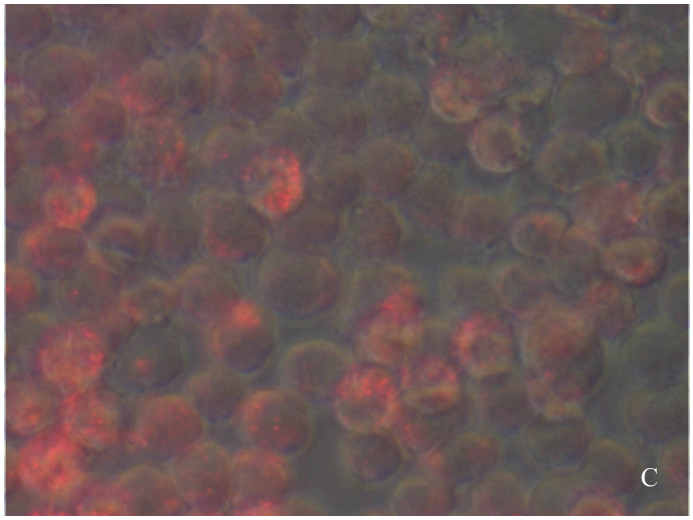


Figure 1
Fluorescence image of RAW267.4 macrophages incubated with Dil labeled liposomes

The degree of liposomes internalization (nmol phospholipids/10^6 cells) was calculated using standard curves obtained from known concentrations of fluorescent liposomes. The results indicate a lower uptake of liposomes containing a phospholipids derivative of PEG (PEG2000-PE) compared to that of conventional liposomes (PC) or PC: PGPH liposomes (data not shown), suggesting that the presence of PEG2000 on surface of the sterically stabilized liposomes hinders their interaction with cells due to the barrier formed by the hydration of the polymer.

Induction of arthritis

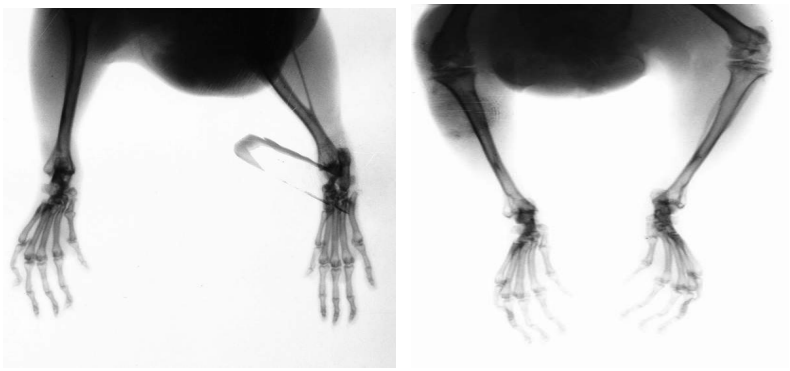
Adjuvant-induced arthritis in rats is a well-established experimental model characterized by proliferative and inflammatory reactions in synovial membranes, producing pain, disability, and eventually destruction of the joints. The following parameters were used for the evaluation of inflammation CFA induced:

- the swelling as a result of primary (injected paw) and secondary inflammation (contralateral non-injected paw) was assessed with plethysmometer device (Ugo Basile, Italy) designed specifically to measure paw swelling in rodents. Evolution of the oedema was measured 7 days and 14 days after administration of CFA.

- the reaction to pain was evaluated after 21 days using an analgesy meter, the classic device to perform paw pressure experiments according to the method of Randall-Selitto. A marked increase in sensitivity to paw pressure was seen in the affected limb.

- the scale of mobility, posture and joint stiffness was determined before the injection of CFA and 14 days after induction of arthritis [4]. Induction of arthritis is confirmed by clinical and behavioural evaluation of the animals, the mobility and posture being significantly lower; a marked increase of the stiffness was recorded.

- the X-ray examination 21 days after administration of CFA. The X-ray absorption is observed mainly on the projection area of the left joint, suggesting an inflammatory process (Figure 2a); the measurement shows an approximately 25% increase in the left joint diameter compared to the right joint.



a b

Figure 2

X-ray examinations of inflamation (a –21 days after administration of CFA; b – after the treatment with hydro-soluble MTX -loaded liposomes)

Haematological parameters

14 days after the last dose of MTX, the number of erythrocytes was not significantly affected, except for a decreasing trend in the groups treated with high-dose of hydrophobic MTX liposomes and low and intermediate doses of MTX injection solution. Moderate decreases of haemoglobin and hematocrite values were observed 14 days after the last MTX dose in the rats treated with low and intermediate doses of MTX–injection solution and hydro-soluble MTX-loaded liposomes and high-dose of hydrophobic MTX liposomes (Figure 3).

The leukocyte formula was impaired, predominantly 7 days after the last MTX dose. Thus, the increase of the percentage of the granulocytes in the groups treated with MTX liposomes has been noticed, indicating that MTX blocked the recruitment of the granulocytes in the swollen synovium and implicitly the aggravation of the inflammatory process. A linear dose-dependent rising (correlation coefficient 0.9923) of the number of granulocytes in the group of rats treated with hydrosoluble MTX liposomes has been revealed (Figure 4).

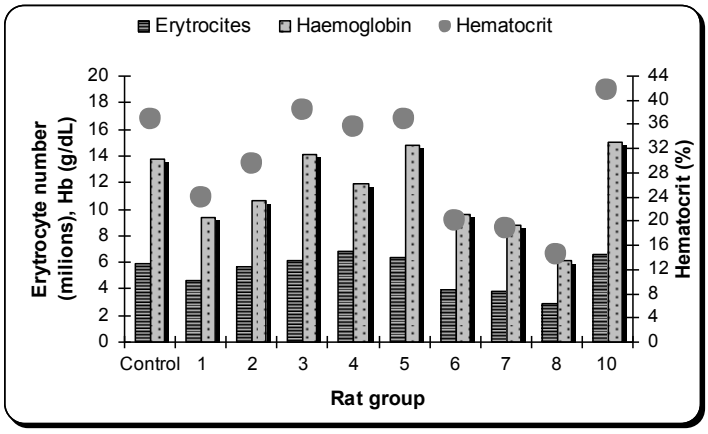


Figure 3

The influence of the MTX treatment on the erythrocyte count, haemoglobin and hematocrite levels, 14 days after the last MTX dose

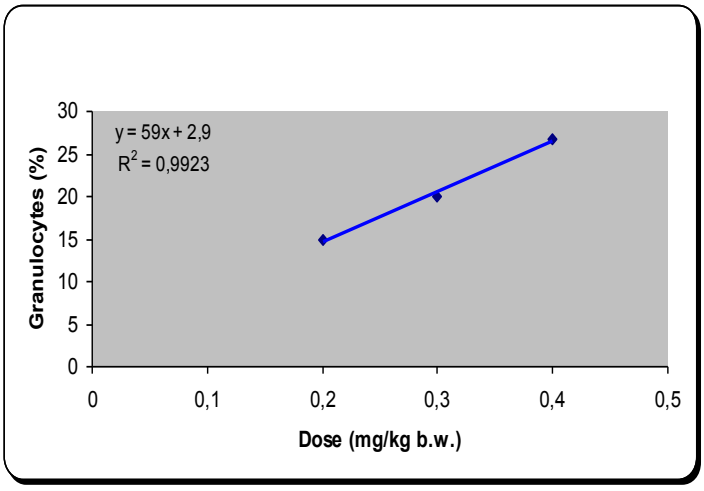


Figure 4

Dose-effect curve of the influence of the hydrosoluble MTX-loaded liposomes treatment on the granulocyte count, 7 days after the last dose

For MTX hydrophobic liposomes, this effect was observed at low and medium doses. The increase of the granulocyte count was associated with a decrease of the monocyte count, indicating a balancing mechanism on the number of phagocytes (Figures 5 and 6).

Lymphocytes population was not significantly affected even after 7 days, or 14 days after the last MTX dose, except for a modest decrease in the groups treated with liposomes, 7 days after the end of treatment (Figure 6).

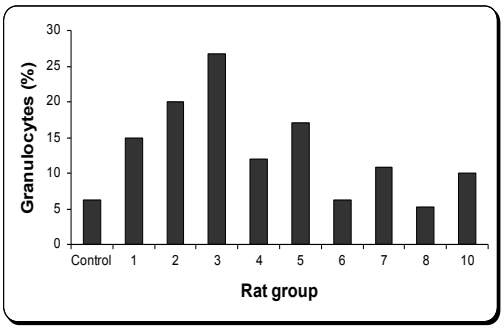


Figure 5
The influence of the MTX treatment on the granulocyte count, 7 days after the last MTX dose

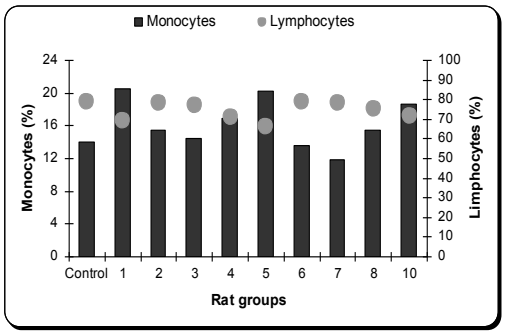


Figure 6
The influence of the MTX treatment on the monocytes and lymphocytes count, 7 days after the last MTX dose

Biochemical parameters

AST and ALT activities were low and irregularly affected. 14 days after the last dose of MTX, a moderate increase in ALT and AST activities in the rat groups treated with the intermediate dose of hydrosoluble MTX liposomes was revealed. A similar behaviour has been noticed for the total serum bilirubin. An increase (approximately 2-fold) in the AST levels was also observed in the groups treated with extreme doses of MTX solution for injection and the median dose of hydrosoluble MTX-loaded liposomes (Figure 7).

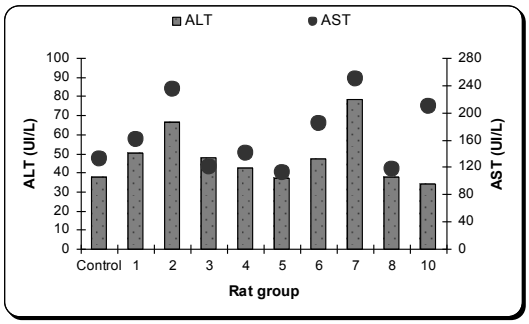


Figure 7
The influence of the MTX treatment on the transaminases activities 14 days after the last MTX dose

Other serum biochemical parameters showed no significant differences. Serum creatinine and urea levels were not significantly altered in any of the test moments, except for the group 7, where a moderate increase was observed.

Conclusions

The results of the haematological and biochemical tests suggest that MTX-loaded liposomes treatment has a reduced toxicity, compared with MTX injectable solution treatment in a murine model of arthritis. The biochemical and haematological results, corroborated with histopathological examination of the liver and kidney (discussed in the note II) indicate a lower toxicity and suggest the safety of MTX in the treatment regimen used.

References

1. Bârcă M**,** Bălălău D, Ilie M, Ciobanu AM, Baconi DL, Spiroiu M, Burcea GTA - Determination of methotrexate encapsulated in liposomes by spectrophotometric methods – *Farmacia*, 2005, 53(6): 30-36.
2. Bârcă M**,** Bălălău D, Ciobanu AM, Olteanu M, Dudau M, Cinteza O - Preparation and characterization of Methotrexate-loaded liposomes. *Ovidius" University Annals of Medical Science – Pharmacy* 1(8), 2004.
3. Bârcă M, Ilie M, Baconi DL, Ciobanu AM, Bălălău D, Burcea GT - Spectrofluorimetric methotrexate assay in human plasma. *Farmacia*, 2010, 58(1): 95-101.
4. Butler SH, Godefroy F, Besson JM, Weil-Fugazza J - A limited arthritic model for chronic pain studies in the rat. *Pain*, 1992; 48(1):73-81.
5. Chan ESL, Cronstein BN - Molecular action of methotrexate in inflammatory diseases. *Arthritis research*, 2002; 4(4):266-73.
6. Foong WC, Green KL - Retention and distribution of liposome entrapped methotrexate injected into normal or arthritic rabbit joints. *J Pharm Pharmacol,* 1988; 40**:**464–468.
7. Hong MS, Lim SJ, Lee MK, Kim YB, Kim KC – Prolonged blood circulation of methotrexate by modulation of liposomal composition. *Drug Deliv*., 2001; 8(4):231-7.
8. Kremer JM -Toward a better understanding of methotrexate. *Arthritis Rheum,* 2004; 50:1370-1382.
9. Trif M, Guillen C, Vaughan DM, Telfer JM, Brewer JM, Roseanu A, Brock JB – Liposomes as Possible Carriers for Lactoferrin in the Local Treatment of Inflammatory Diseases. *Exp Biol Med,* 2001; 226(6):559–564.
10. Van den Hoven JM, Van Tomme SR, Metselaar JM, Nuijen B, Beijnen JH, Storm G - Liposomal drug formulations in the treatment of rheumatoid arthritis. *Mol Pharm*, 2011; 8(4):1002-15.
11. Williams AS, Camilleri JP, Goodfellow RM, Williams BD – A single intra-articular injection of liposomally conjugated methotrexate suppresses joint inflammation in rat antigen-induced arthritis. *Br. J. Rheumatol,* 1996; 35(8):719-24.

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