We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,300

Open access books available

172,000

International authors and editors

190M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Methotrexate Liposomes - A Reliable Therapeutic Option

Anne Marie Ciobanu, Maria Bârcă, Gina Manda,
George Traian Alexandru Burcea Dragomiroiu and
Daniela Luiza Baconi

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/intechopen.68520>

Abstract

Liposomes were proposed as drug vector systems in the treatment of many diseases. The following characteristics recommend the liposomes as attractive candidates for drug transportation: solubilisation, duration of action, targeting potential and internalisation. Methotrexate, a folate antagonist, was originally developed as an antineoplastic agent and subsequently used in inflammatory and/or immunosuppressive diseases. Its side effects have led researchers to direct their efforts to reduce toxicity, while maintaining efficacy of methotrexate. Liposomes with methotrexate as such, as well as its disodium salt, were prepared using two methods. The liposomes were characterized in terms of structure, size, degree of poly-dispersion and encapsulation efficiency. The effect of methotrexate incorporated in liposomes has been investigated *in vitro* on human lymphoblastic cell line K562. Methotrexate incorporated into liposomes moderately reduces the proliferation of K562 cells, but significantly inhibits RNA synthesis. The cellular activation is probably the main target of the drug and not the neoplastic proliferation of cells. The methotrexate liposomes exhibited significant anti-inflammatory activity and showed reduced toxicity. Given that the encapsulating of the drug in vector systems may result in the increasing concentration at the site of action, the methotrexate liposomes represent a targeted therapy with an optimized therapeutic efficacy—risk toxicity ratio.

Keywords: liposomes, methotrexate, rheumatoid arthritis

---

1. Introduction

Liposomes have been proposed as drug vector systems in the treatment of many diseases. Among the drugs proposed to be encapsulated in liposomes, remarkable are

drugs used in anti-fungal therapy (amphotericin B, nystatin, econazole), in anticancer therapy (doxorubicine, daunorubicin, methotrexate (MTX), cytarabine, vincristine, paclitaxel, mitoxantrone), in the treatment of asthma (albuterol) and in the treatment of some inflammatory diseases (clodronate, methotrexate, lactoferrin), in anti-viral therapy (e.g. for the induction of interferon production), as well as for radio diagnostic purpose (indium-111) [1–9].

The following characteristics recommend the liposomes as attractive candidates for the drug transportation: solubilisation (the liposomes can solubilize lipophilic drugs that would be difficult to administer intravenously), duration of action (the liposomes function as a micro-reservoir which gradually release the drug into the body), targeting potential (by coupling of some ligands on the liposomes surface, it can direct a drug to a specific target) and internalisation (the liposomes interact with the target cell and may be able to promote intracellular transport of some molecules).

Because they are usually prepared from lipids of natural origin, biodegradable and non-toxic, liposomes are useful as drug vector systems that can reduce systemic toxicity [10]. Side effects associated with anti-tumour drugs administered in conventional dosage forms can be reduced by encapsulating them in liposomes. Therefore, the encapsulation of medicines in liposomes is a tool to increase the therapeutic index by reducing the drug toxicity and targeting the specific cells [11].

Although liposomes were first described by Alec D Bangham in 1965, the first liposomal drug product was approved by the Food and Drug Administration (FDA) in 1995 and contains the anticancer drug doxorubicin (Doxil®, doxorubicin hydrochloride liposome injection) [12]. Currently, several liposome-based drugs containing antifungal drugs (amphotericin B, Ambisome®, Abelcet®, Amphotec®), anticancer drugs (daunorubicine, Daunoxome®; doxorubicine, Doxil®, Lipo-dox®, Myocet®; cytarabine, Depocyt®) and photosensitizer for photodynamic therapy (verteporfin, Visudyne®) are approved for clinical use, mainly for intravenous administration [13].

Methotrexate, a folate antagonist, was originally developed as an antineoplastic agent and subsequently used in inflammatory and/or immunosuppressive diseases [14]. Among the cytotoxic agents, methotrexate has been widely used as an immunosuppressant in autoimmune diseases [15]. In 1951, the proposal for the use of methotrexate in the treatment of rheumatoid arthritis was based on its inhibitory effect on the proliferation of lymphocytes and other cells responsible for inflammation of the joint [16]. However, by 1980 there have not been reported and published any clinical studies regarding the use of methotrexate in rheumatoid arthritis. MTX is currently accepted as the most effective and well-tolerated disease-modifying anti-rheumatic drug (DMARD) for rheumatoid arthritis [17, 18] with certain effects of slowing the progression of the disease and reducing mortality rate [19]. The broad spectrum of side effects and the relatively high frequency of them have led researchers to direct their efforts to reduce toxicity, while maintaining at the same time the therapeutic efficacy of methotrexate. In this regard, both alternative routes of administration (especially in the treatment of inflammatory

diseases) and new pharmaceutical formulations with methotrexate were investigated. It has been suggested that the anticancer drugs formulated in liposomes would be the long-awaited 'magic pill' for cancer therapy, due to their ability to selectively accumulate in tumours; at the same time, toxicological studies indicate that encapsulation in liposomes provides protection against the majority of the adverse effects of chemotherapy drugs. On the other hand, liposomes were shown to give an effective and appropriate delivery of anti-rheumatoid drugs to the synovial fluid [20].

Liposomes with methotrexate as such (further named 'hydrophobic methotrexate' liposomes), as well as its disodium salt (further named 'hydrosoluble methotrexate' liposomes), were prepared using two methods: the lipid film hydration method and reverse-phase evaporation method (REV). The liposomes were characterized in terms of structure, size, and degree of poly-dispersion and encapsulation efficiency. Methotrexate incorporation into liposomes has been achieved by passive loading method which encapsulates the active compound during liposome formation or in a stage of preparation when the liposomal structure is very fluid.

The effect of methotrexate incorporated in liposomes has been investigated *in vitro* on human lymphoblastic cell line K562.

The effects of short-term therapy with methotrexate incorporated into the liposomes have also been demonstrated in an *animal model of rheumatoid arthritis*.

2. Preparation of methotrexate liposomes

Liposomes are phospholipid vesicles made up of one or more concentric phospholipid bilayers alternating with layers of aqueous. Phospholipids are a very attractive transport way of drugs and other molecules not only because they are able to form lamellar phases but also because they are natural components of cell membranes having low allergenic potential; they can be metabolized in a manner similar to the endogenous phospholipid membrane and have the advantage of structural variability which can be used to modify the physical properties of liposomes so as to increase selectivity for target organ.

Liposomal properties depend on both the choice of phospholipids and the addition of sterols, particularly cholesterol, and glycolipids [21].

Over time, the size, number of lamellae and the characteristics of the lipid bilayer were handled depending on the purpose of the liposome. Thus, conventional liposomes, sterically stabilized liposomes ('stealth' liposomes), cationic liposomes or targeted liposomes (by coupling ligands to the surface) have been developed. Sterically stabilized liposomes, undetectable ('stealth'), contain lipid derivatives of a polymer (polyethylene glycol, PEG) inserted into the lipid bilayer, which gives them the advantage of the enhanced circulation times.

Conventional liposomes and sterically stabilized liposomes with the following compositions were prepared:

1. Phosphatidylcholine (PC)
2. Phosphatidylcholine:cholesterol (PC:CH)
3. Phosphatidylcholine:cholesterol:polyethylene glycol-2000-phosphatidylethanolamine (PC:CH:PEG2000-PE).

Liposomes are used to encapsulate both hydrophobic and hydrosoluble drugs within the bilayer and the aqueous core, respectively. Consequently, both methotrexate as such (hydrophobic methotrexate liposomes) and its disodium salt (hydrosoluble methotrexate liposomes) were encapsulated in liposomes. Methotrexate incorporation into liposomes has been achieved by passive loading method which encapsulates the active compound during liposome formation or in a preparation stage when the liposomal structure is very fluid [22].

The following weight ratios between the lipid phase and the active substance methotrexate were used: PC:MTX 10:1; PC:CH:MTX 10:2:1 and PC:CH:MTX 10:1:1 for conventional liposomes and PC:CH:MTX:PEG2000 10:1:1:1 for sterically stabilized liposomes [23].

Also, 'control liposomes' (or empty liposomes, or liposomes unloaded with methotrexate) were prepared using the following compositions: PC or PC:CH (10:1 and 10:2) for conventional liposomes and PC:CH:PEG2000-PE (10:1:1) for sterically stabilized liposomes. In order to track the cellular internalisation, we prepared the 'control liposomes' sterically stabilized with the composition PC:PGPH (polyglycerol 12-hydroxystearic acid ester) (10:1).

Two methods of preparation were used: the lipid film hydration method [24] and reverse-phase evaporation method [25–27].

2.1. Lipid film hydration method

The mechanism of liposome formation by lipid film hydration method, combined with extrusion, consists of the following sequence of steps: initially, thin lipid film is hydrated and lipid layers become fluid, then, hydrated lipid lamellae are detached and self-closed, to form large multilamellar vesicles. In order to reduce the size, extrusion of the liposomes is performed, which determines the conversion of multilamellar liposomes in unilamellar liposomes.

The lipid film hydration method was used for the preparation of liposomes with hydrophobic methotrexate. Soybean lecithin and cholesterol were dissolved in chloroform-methanol (2:1, v/v), then the active substance is added and stirred to mix. The organic solution is then subjected to evaporation under reduced pressure in the rotary evaporator to remove the organic solvent. Thin lipid film displayed on the wall's balloon is hydrated by adding pH 7.4 phosphate buffer. The resulting dispersion is kept at rest for 48 h.

2.2. Reverse-phase evaporation method

Reverse-phase evaporation method allows to obtain large unilamellar liposomes (or large unilamellar vesicles, LUV), with a significant aqueous compartment. In this process, phospholipids are dissolved in an organic solvent or in a mixture of organic solvents. Then, the aqueous phase is added to the organic phase. At this stage, phospholipids are placed at the interface between two immiscible phases. A W/O emulsion is formed by ultra-sonication or magnetic stirring. The success of emulsification is a fundamental condition to obtain unilamellar liposomes with high encapsulation capacity. The removal of the solvent by evaporation leads to the closeness of the micelles and, consequently, to the formation of a gel emulsion. During this step, the micelles are forming monolayers surrounding aqueous compartments and aggregate to form a compact gelled network. During the next stage, the pressure is reduced to promote the complete evaporation of the organic solvent, at which the destructurement of the gel occurs and the monolayers are getting closer to form liposomal bilayers. This process can be accelerated by shaking the solution using a vortex.

For the preparation of hydrophobic methotrexate liposomes, soya lecithin and cholesterol or PEG2000-PE were dissolved in chloroform-methanol (2:1, v/v), then the active substance is added and stirred to mix. Equal volumes of the organic solution and phosphate buffer solution pH 6 were mixed under magnetic stirring until a W/O emulsion was obtained. Organic solvents were then evaporated to obtain a gel emulsion. After the destructuring of the gel, pH 6 phosphate buffer was added and stirring was continued until the liposomal dispersion is formed.

For the preparation of hydrosoluble methotrexate liposomes, soya lecithin and cholesterol or PEG2000-PE were dissolved in chloroform-methanol (2:1, v/v), and equal volumes of the organic solution and sodium salt of methotrexate were mixed under magnetic stirring until a W/O emulsion was obtained. Organic solvents were then evaporated to obtain a gel emulsion. After the destructuring of the gel, pH 7.4 phosphate buffer was added and stirring was continued until the liposomal dispersion is formed.

2.3. Reducing the size of liposomes and increasing the uniformity of their size by extrusion

Since the formed liposomes are heterogeneous in size, a uniform dispersion is obtained by extrusion. For particle size reduction, liposome dispersions were passed 10 times through a polycarbonate membrane (Whatman Nucleopore® Tch Track Membrane-E) with a pore diameter of 3 μm and then through a membrane with a pore diameter of 100 nm. The pre-filtration through a filter membrane with larger pores (3 μm) is necessary to prevent clogging of the membrane. In the case of sterically stabilized liposomes, as they have been used in studies *in vivo*, extrusion was carried out through a polycarbonate membrane with a 100-nm pore size.

3. Characterization of methotrexate liposomes

The liposomes were characterized in terms of structure, shape, size and degree of poly-dispersion and methotrexate encapsulation efficiency.

Characterisation of the obtained liposomes was pursued as follows:

- The visualisation and the determination of the type of liposomes using enhanced video microscopy (VEM) and transmission electron microscopy (TEM);
- The determination of the size and size distribution of liposomes by dynamic light-scattering technique (DLS);
- The determination of the encapsulation rate and the determination of the content of active substance in liposomes.

In addition to the general methods for liposomes characterisation, intracellular liposomes transport was studied by fluorescence microscopy, and quantification of cell internalisation of 'control liposomes' was studied by fluorimetric technique.

3.1. The visualisation and the type of liposomes

Microscopy is a method for observing liposomal dispersion and determining the shape and the size of liposomes. The ability of this method to directly visualize colloidal structures in real time allows to observe dynamic changes in the number and size of the vesicles and also offers the possibility of discovering new structures.

Examination by enhanced video microscopy showed in particular the shape, size and state of dispersion, but no information on liposome structure was obtained using this technique.

VEM images of methotrexate hydrophobic liposomes prepared by the two methods, the hydration of the lipid film and reverse-phase evaporation, are shown in **Figures 1 and 2**.

Figure 1. VEM images of hydrophobic MTX liposomes prepared by the method of hydration of the lipid film: PC:MTX = 10:1 (a), PC:CH:MTX = 10:1:1 (b), PC:CH:MTX = 10:2:1 (c).

Hydrophobic methotrexate liposomes prepared by both methods were unilamellar and poly-disperse. The presence of cholesterol leads to a decrease of the average diameter of the liposomes. The ratio PC:CH influences the size of liposomes.

Hydrosoluble methotrexate liposomes were prepared only by the reverse-phase evaporation method due to higher encapsulation of methotrexate. In this case, the same change in size of the liposomes in the presence of cholesterol was observed (**Figure 3**).

Variation of liposomes size depending on the lipid layer composition can be explained by the fact that, at the working pH, the phosphatidylcholine polar groups are charged with negative electric charges, which cause electrostatic repulsion between them with the formation of large vesicles; cholesterol, due to its amphiphilic properties, is inserted between phosphatidylcholine molecules shielding the electrostatic repulsion between the polar groups and thereby increase the radius of curvature of the bilayer.

Considering under micron size of the obtained vesicles, transmission electron microscopy was also used. The suspension of liposomes was analysed using the negative staining electron

Figure 2. VEM images of hydrophobic MTX liposomes prepared by reverse-phase evaporation method: PC:MTX = 10:1 (a), PC:CH:MTX = 10:1:1 (b).

Figure 3. VEM images of hydrosoluble MTX liposomes prepared by reverse-phase evaporation method: PC:MTX = 10:1 (a), PC:CH:MTX = 10:1:1 (b).

microscopy with 1% phosphotungstic acid. Liposomal dispersions with methotrexate (PC:CH:MTX = 10:1:1) were stored at a temperature of 3–5°C and monitored for 3 days (after 48 h, **Figure 4a**, and after 72 h, **Figure 4b**). After 72 h of preparation, TEM images showed a reversible coagulation process, unaccompanied by the membrane destruction. This process is similar to emulsion-clotting process which does not cause the emulsion destruction. The presence of electrical charges on the surface of liposomes explains their electrostatic stabilisation.

The encapsulation of hydrosoluble methotrexate in the internal aqueous liposomal medium results in larger liposomes than those obtained in the absence of MTX (**Figure 5**).

The same effect was observed for liposomes loaded with hydrophobic MTX prepared using pH = 7.4 buffer (**Figure 6**) due to the solubilisation in the aqueous phase of a part of MTX initially encapsulated in liposome membrane. Increasing the size of the liposomes in the presence of methotrexate can be explained also by the osmotic pressure. Due to the hydrophobicity of the lipid bilayer and taking into account that at pH 7.4, both PC and MTX are charged with electrical charges of the same sign, methotrexate diffusion through liposomal membrane is prevented. The pressure difference on both sides of the liposomal membrane occurs due to the difference in the concentration of methotrexate in the inner aqueous phase and in the dispersion medium.

Figure 4. TEM images of the hydrosoluble MTX liposomes, 48 h (a) and 72 h (b) after preparation.

Figure 5. VEM images of 'control liposomes' (PC:CH) (a) and hydrosoluble MTX liposomes (PC:CH:MTX) (b).

In order to reduce the solubilisation of hydrophobic methotrexate in the aqueous medium at pH 7.4, and to increase the efficiency of encapsulation, hydrophobic methotrexate liposomes were prepared by reverse-phase evaporation method, using a pH 6 buffer solution as a dispersion medium. TEM images of the liposomes prepared as such are shown in **Figure 7**.

The results of microscopic examination suggested that the presence of methotrexate in the liposome membrane does not affect the size of the liposomes. It would be expected that the presence of methotrexate in bilayer increases the size of the liposomes. The molecules of organic acids with odd number of carbon atoms are not flat, but have a twisted structure and are not centre-symmetrical, but have a binary axis of symmetry. Carboxyl groups are inclined at an angle of 60° relative to each other and 30° to the plane of zigzag chain of carbon atoms [28]. Given this structure of methotrexate, inserting it between molecules PC would have been expected to result in an increase of lecithin vesicle size.

The methotrexate molecule is not placed between the lecithin molecules due to its pronounced hydrophobic character but it is encapsulated in the hydrophobic region of the bilayer leading

Figure 6. TEM images of 'control liposomes' (PC) (a) and hydrophobic MTX liposomes prepared with pH 7.4 buffer (PC:MTX) (b).

Figure 7. TEM images of 'control liposomes' (PC) (a) and hydrophobic MTX liposomes prepared with pH 6 buffer (PC:MTX) (b).

eventually to an increase in the thickness of the bilayer made, but not in an increase of liposomes size.

In order to reduce the liposomes size and to increase the uniformity of their size, liposomal poly-dispersion was extruded by passing them through polycarbonate membranes with different pore diameters: first membrane with a pore diameter of 3 μm , then a membrane with a pore diameter of 100 nm in the case of sterically stabilized liposomes. Liposomes were extruded to increase their stability and in order to decrease the size under the diameter of capillaries for intravenous administration. Examples of TEM images of the MTX liposomes before and after extrusion are shown in **Figures 8**.

3.2. The determination of the size and size distribution of liposomes

To determine the size and size distribution of the liposomes, dynamic light-scattering technique was used. This technique can be applied to systems in which the average diameter is less than 1 μm . The advantage of the DLS to electron microscopy is that information can be obtained quickly (minutes) and is less expensive. To determine the size distribution of the liposomes by this method, a NICOMP 270 DLS Submicron Particle Sizer (Pacific Scientific® Hiac/Royoco Instruments Division) was used.

The decrease in liposomes diameter when the cholesterol was added in the lipid phase observed by microscopic techniques was confirmed by the results of the DSL determinations. A decrease of approximately 50% in the mean diameter of liposomes was observed for PC:CH formula, from 2502.6 to 1450.2 nm.

The increase of liposomes size after hydrosoluble methotrexate encapsulation observed by VEM and TEM techniques (**Figure 5**) is supported by the DLS results (**Figure 9**). It is noted that the average diameter of the PC:CH:MTX liposomes (4893.2 nm) is superior to that obtained for PC:CH liposomal dispersion (2502.6 nm).

3.3. The determination of the encapsulation rate

Methotrexate liposome encapsulation efficiency was estimated by the determination of loading yield. Load yield is the ratio of the amount of active substance encapsulated in

Figure 8. TEM images of PC:CH:MTX liposomes before (a) and after (b) extrusion.

Figure 9. Histogram of PC:CH:hydrosoluble MTX liposomal dispersion.

liposomes and the initial amount of active substance and is calculated using the following formula:

$$R_i = \frac{C_f}{C_0} \times 100 \quad (1)$$

where C_f is the concentration of active substance in the liposome dispersion after the removal of unloaded active substance, and C_0 is the concentration of active substance in the lipid mixture used for liposomes preparation.

In order to determine the concentration of active substance in the liposome dispersion (C_f), the removal of the unloaded active substance was done by dialysis for hydrosoluble methotrexate liposomes and by Sephadex gel filtration for hydrophobic methotrexate liposomes.

The dialysis process was monitored by the UV spectrophotometric determination of the methotrexate in dialysate ('washing water') in order to confirm that all the unloaded methotrexates were removed (Figure 10).

After the removal of unloaded active substance, the liposomal dispersion was subjected to lysis with Triton X-100 and methotrexate was quantified by high-performance liquid chromatography (HPLC).

The efficiency of methotrexate encapsulation, measured by loading yield, was similar for hydrosoluble methotrexate liposomes and hydrophobic methotrexate liposomes. However, in the case of hydrophobic methotrexate liposomes slightly higher loading yields were obtained when cholesterol is added in the lipid layer.

Figure 10. UV-spectrum of methotrexate in dialysate ('washing water').

The electric charge of liposomes is a predictive factor of their lifecycle. Preliminary studies performed on liposomes showed that the presence of lipids with negative electric charge leads to a reduced elimination of the encapsulated substance. The low permeability for hydrosoluble methotrexate of the anionic liposomal membrane explains the high encapsulation efficiency obtained. At the same time, the presence of structures with a large interfacial area per volume unit (cubosomes and hexasomes) in the colloidal dispersions obtained using REV causes a higher encapsulation of hydrophobic substances.

3.4. The determination of content of active substance in liposomes

For the quantitative determination of methotrexate, the liposomal dispersion was subject to ultracentrifugation and the active substance was determined by HPLC after the liposomes lysis with Triton X100. Chromatographic conditions were as follows: HPLC Millenium Waters, Spherisorb 5 ODS 250×4.6 mm column, mobile phase 5% tetrahydrofuran in 0.05 M sodium dihydrogen orthophosphate buffer (pH 4.85), flow rate 1.0 mL/min, 20 μ L injected volume, UV detection at 313 nm. The concentration of methotrexate in the liposomal dispersion was calculated based on the methotrexate peak area and the obtained calibration curve. The selectivity of the method for the determination of methotrexate in liposomes was demonstrated by analysis of MTX-unloaded liposomes ('control liposomes'). The lipids contained in the liposome membrane do not interfere with methotrexate.

The average methotrexate concentration measured was 196 mg/mL for water-soluble methotrexate liposomes (Figure 11) and 200 mg/mL for hydrophobic methotrexate liposomes (Figure 12).

3.5. Intracellular transport of liposomes studies

Studies of the interaction between liposomes and cells are of particular importance in order to develop liposomes as vectors with high efficiency for delivering drugs to cells. Therefore, the development of liposomal systems as drug carriers requires detailed understanding of interaction mechanisms between cells and these transporters. Some studies have indicated that the *in vitro* uptake of the liposomes depends on the cell type [29, 30], but the factors that are involved in this uptake are not fully understood. In general, it is believed that the uptake of the liposomes is mediated by nonspecific adsorption to the cell surface [31].

Figure 11. Chromatogram of hydrosoluble methotrexate liposomes.

Figure 12. Chromatogram of hydrophobic methotrexate liposomes.

Given the importance of the liposomes uptake by macrophages to the elimination from the bloodstream after liposomes intravenous injection, we studied the uptake and the quantification of internalisation of liposomes with different compositions of lipid bilayer by macrophages from tumour line RAW267.4.

The internalisation of the following types of unloaded liposomes was studied: conventional liposomes containing phosphatidylcholine, steric-stabilized liposomes containing phosphatidylcholine, cholesterol, polyethylene glycol 2000 phosphatidylethanolamine

(PC:CH:PEG2000-PE) and PC:PGPH. The cells were incubated for 2 h at 37°C with the Dil-labelled liposomes.

3.5.1. Visualisation of the liposomes internalisation

The internalisation of the methotrexate-unloaded liposomes by macrophages of murine tumour line RAW267.4 was visualized by fluorescence microscopy, using 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil) as a lipophilic tracer. Images were acquired using a Nikon microscope, in phase contrast or epifluorescence, with a filter that allows 530-nm excitation and observation of emitted fluorescence at 580 nm. Intracellular point-like fluorescence is observed, indicating that the liposomes are internalized (**Figure 13**) [32].

3.5.2. Quantification of liposomes internalisation

Quantitative estimation of the ability of RAW 264.7 tumour macrophages to uptake of various types of liposomes was assessed by fluorimetric measurements. For this purpose, cells were incubated for 2 h at 37°C with methotrexate-unloaded liposomes, labelled with fluorescent Dil tracer (1 μmol liposomes/ 10^6 cells). After incubation, the liposomes bounded to cell surface were removed by washing with cold PBS buffer.

After washing with trypan blue solution (for complete quenching of extracellular fluorescence), the emitted fluorescence was measured at 580 nm after excitation at 530 nm of samples, using a TECAN spectrofluorimeter. The degree of internalisation of the liposomes (phospholipid nmol/ 10^6 cells) was calculated using the standard curve obtained from known concentrations of fluorescent liposomes.

The results showed a low uptake of liposomes containing polyethylene glycol-2000-phosphatidylethanolamine (PEG2000-PE) compared to that of conventional liposomes (PC) or PC-PGPH liposomes (**Figure 14**). One possible explanation would be that the presence of PEG2000 on the surface of steric stabilized liposomes hinders their interaction with cells through the barrier formed by hydration of the polymer [32].

Figure 13. Phase contrast images (A) and fluorescence images (B) obtained in RAW 264.7 macrophages incubated for 2 h at 37°C with PC liposomes, labelled with Dil (20 $\times$ objective).

Figure 14. Internalization of PC, PC:PGPH, PC:CH:PEG-PE liposomes by murine macrophages RAW 264.7 after the incubation with 1 μmol liposomes/ 10^6 cells Phase contrast images (A) and fluorescence images (B) obtained in RAW 264.7 macrophages incubated for 2 h at 37°C with PC liposomes, labelled with Dil (20 $\times$ objective).

4. *In vitro* effects of MTX liposomes

The immunosuppressive action exerted *in vitro* by MTX-loaded liposomes was studied.

We compared the effect of hydrosoluble and hydrophobic MTX liposomes (dispersion 200 mg MTX/mL) and MTX solution for injection (concentration 200 mg MTX/mL) on the proliferative capacity of human lymphoblastic cells K562. The preparation and characterisation of MTX-loaded liposomes are presented in Sections 2 and 3.

The human lymphoblastic K562 cell line, purchased from ECACC (the European Collection of Cell Cultures), maintained by *in vitro* cultivation, has been used. Colchicine (standard microtubule disrupter) at a concentration of 10 μM was used as an inhibitor of cell metabolism. Cell proliferation was measured by the MTS reduction test by using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (detects the number of viable cells and, consequently, cell multiplication) (Promega Corporation). The cell activation/proliferation was measured by the tritium-labelled uridine (3H-Urd) incorporation test which detects RNA synthesis requiring uridine incorporation via the salvage pathway of nucleotide biosynthesis. The cellular membrane integrity was indirectly evaluated as lactate dehydrogenase (LDH) release, by using Cytotox96® NonRadioactive Cytotoxicity Assay kit (Promega Corporation).

The effect exerted by MTX was calculated as the ratio between the values obtained for MTX and the control value.

Experimental data indicate that MTX solution for injection inhibits neoplastic multiplication (data not shown) and RNA synthesis (**Figure 15**) in lymphoblastic K562 cells, without notably disturbing membrane integrity evaluated as LDH release.

Figure 15. The effect exerted *in vitro* by MTX solution for injection on RNA synthesis by K562 cells.

PC and PC:CH liposomes do not alter significantly the multiplication of K562 cells, but, when loaded with hydrophobic MTX, they tend to hold down the proliferation of tumour cells (**Figure 16**). These results are confirmed by those obtained in the evaluation of RNA synthesis by the tritium-labelled uridine radionuclide technique. Thus, while the unloaded liposomes (PC and PC:CH) tend to stimulate the RNA synthesis, the corresponding MTX-loaded liposomes clearly induce RNA synthesis arrest (**Figure 17**).

The increase of the quantity of liposomal dispersion (treating the cells with a double amount '2×' of liposomal dispersion) does not significantly influence the MTX inhibitory effect. It is worth noticing that hydrosoluble MTX effect (as solution for injection) is also independent on the drug concentration in the range of 0.001–10 µg/mL (data not shown).

Figure 16. The effect exerted *in vitro* on the multiplication of K562 cells by liposomes loaded with hydrophobic MTX, compared to unloaded liposomes. Cell proliferation has been evaluated by MTS reduction test.

Figure 17. The effect exerted *in vitro* on the multiplication of K562 cells by liposomes loaded with hydrophobic MTX, compared to unloaded liposomes. Cell proliferation has been evaluated by the tritium-labelled uridine incorporation test. The effect has been assessed comparatively to colchicine antimitotic standard.

The inhibitory effect of hydrosoluble or hydrophobic MTX-loaded liposomes on K562 cell multiplication is comparable. However, hydrophobic MTX-loaded liposomes have a little more intense inhibitory effect compared to hydrosoluble MTX-loaded liposomes.

The results indicate that hydrophobic MTX loaded in liposomes tends to restrain the tumoural multiplication of K562 cells and clearly inhibits RNA synthesis, suggesting that activation events are primarily the target of the drug, and not the neoplastic proliferation of lymphoblasts. In addition, the hydrophobic form of MTX loaded in liposomes acts similar to the hydrosoluble one.

5. Investigation of the effects of short-term therapy with methotrexate incorporated into the liposomes in an animal model of rheumatoid arthritis

Rheumatoid arthritis is a chronic, complex, autoimmune disease with plurifactorial etiology. It is characterized by hyperplasia of the synovium of the joint cartilage [33], the infiltration of the synovial cavity with inflammatory cells [33, 34], the presence of autoreactive lymphocytes [35–37] and antibodies with different specifications [38], events that culminates in the gradual erosion of the cartilage/bone and a number of serious extra-articular manifestations [39].

Methotrexate is one of the most widely used disease-modifying anti-rheumatic drugs (DMARDs) in the treatment of rheumatoid arthritis. Although the precise mechanism of action of folate antagonist MTX in the treatment of rheumatoid arthritis is yet unclear [40], the effectiveness of methotrexate is associated with its cytotoxic and anti-inflammatory effects. Clinical and experimental evidence sustain that low-dose MTX has anti-inflammatory effects and a subtle immunomodulatory action [16, 41]. Low dose of methotrexate, orally administered, weekly, effectively suppresses inflammation in rheumatoid arthritis [42]. However,

systemic toxicity, manifested for instance by stomatitis, nausea, bone marrow depression and liver damage, may limit oral administration of the drug [43].

Methotrexate has also been administered to control intra-articular synovitis in the joints of arthritic patients, but the results have been disappointing due to rapid clearance of the drug from the joint [44].

In order to localize the drug to the site of action and reduce the systemic toxicity, the use of liposomes or polymeric microparticles as carriers for drug delivery systems synovial space was proposed.

Effects of short-term therapy with methotrexate incorporated into the liposomes have also been investigated in an experimental model of arthritis-type inflammation-induced with Freund's adjuvant.

Freund's adjuvant-induced arthritis in the rat is one of the most important experimental models of immune chronic inflammation, with pharmacological relevance in human rheumatoid arthritis. It is most commonly used experimental model for rheumatoid arthritis in screening programmes aimed at finding new-arthritic inflammatory drugs [45].

In a Wistar rat model of arthritis (adjuvant Freund induced), the therapeutic effect and toxicity of MTX as solution for injection or hydrosoluble MTX and hydrophobic MTX-loaded liposomes have been studied [32, 46]. Three different doses of MTX preparations have been administered (i.v.) weekly for 21 days: 0.2 mg/b.w., 0.3 mg/b.w and 0.4 mg/b.w.

The induction of arthritis with Freund's adjuvant and its characterisation was based on the evaluation of the primary oedema due to inflammation (injected paw) and the secondary inflammation (paw contralateral, not injected), using a plethysmometer device (Ugo Basile, Italy) 7 days and 14 days after administration of CFA. The threshold pain response was also assessed after 21 days using an analgesy metre, according to the method of Randall-Selitto [47]. Before the injection of Freund's adjuvant, and 7, 14 and 21 days after induction of arthritis the mobility scale, posture and joint stiffness were evaluated [48]. In addition, the X-ray examination 21 days after administration of CFA has been performed to evaluate the inflammation CFA induced.

The effect of MTX treatment was assessed as threshold of pain sensitivity (Randal-Sellito test) 7, 14 and 21 days of MTX administration, as well as by radiological evaluation 21 days of MTX administration.

The induction of arthritis by Freund adjuvant was confirmed by the statistical results [*t*-Student test and analysis of variance (ANOVA)] of the inflammatory oedema assessment, the clinical assessment and the behaviour of animals (with mobility and posture significantly lower and a marked increase of stiffness), as well as the radiological evaluation of the joints (symmetric arthritogenic disturbances were present after 21 days) [32]. In addition, a marked increase in sensitivity to paw pressure was seen in the affected limb.

A dose-dependent reduction of pain sensitivity in all groups of animals treated with MTX has been shown. In addition, the intensity of the therapeutic effect increased during

treatment (the marked effect has been observed after 21 days of MTX treatment). The effect of MTX treatment has been assessed from the baseline values of the pain sensitivity (determined by Randal-Sellito test) and has been calculated after 7, 14 and 21 days of MTX treatment.

The results of the study indicated that the therapeutic effect of MTX liposomes is superior to that of MTX solution for injection. At the highest dose administered (0.4 mg/kg), the therapeutic effect of hydrosoluble and hydrophobic MTX liposomes is comparable, while at intermediate and low dose, the effect of hydrophobic MTX liposomes is higher than that of the hydrosoluble MTX liposomes. Based on the linear relationships between the MTX effect and log *D* (dose), ED₅₀ values have been calculated (**Table 1**). Thus, the lowest efficacious doses of MTX were obtained at all times of the treatment for the MTX-loaded liposomes. The results are in agreement with recent data indicating that MTX encapsulated in liposomes, in contrast to free and generic MTX, proved to have a higher anti-inflammatory and anti-angiogenic efficacy in antigen-induced arthritis model in female C57/Bl6 mice [49].

The immune status of animals was evaluated 7 and 14 days after treatment discontinuation by the following parameters: number of peripheral leucocytes, relative weight of spleen (the ratio spleen weight/body weight), number of splenocytes and the activation potential of splenocytes *in vitro* treated with polyclonal mitogen concanavalin A (ConA) determined by the tritium-labelled uridine incorporation test [50].

Hydrosoluble MTX liposomes particularly tend to enhance the peripheral granulocytes percentage on behalf of the monocyte proportion. Liposome-targeted MTX induces a drop of the monocytes percentage at lower doses than the MTX solution for injection [32]. The mentioned effect ceases 14 days after therapy discontinuation. The effect is less obvious in the case of hydrophobic MTX liposomes. While peripheral monocytes percentage decreases shortly after the withdrawal of the therapy with MTX liposomes, a tendency of up-regulation was noticed 14 days after. Peripheral leukocytes react to lower doses of MTX loaded in liposomes, as compared to MTX solution for injection.

Animals treated with MTX liposomes present lower values of the relative spleen weight. This effect is reversible after 14 days since therapy withdrawal. Similar effects are exerted only by

MTX treatment	ED ₅₀ (mg/kg)		
	7 days of treatment	14 days of treatment	21 days of treatment
Hydrophobic MTX liposomes	0.338	0.272	0.258
Hydrosoluble MTX liposomes	0.363	0.337	0.285
MTX solution for injection - solution	-	0.423	0.387

Table 1. ED₅₀ of MTX, determined on the base of pain sensitivity for each group of animal.

high doses of MTX solution for injection. The clinical significance of the registered decrease of the relative spleen weight is unclear.

The intermediate doses of MTX-loaded liposomes increase the number of spleen leukocytes, probably on behalf of the peripheral ones.

All MTX formulations induce *in vivo* activation of splenocytes, but only MTX-loaded liposomes restrain the activation potential of splenocytes to exogenous polyclonal mitogens. Seven days after therapy withdrawal, splenocytes are basically activated in the absence of exogenous

Figure 18. The effect exerted *in vivo* by MTX on the proliferation capacity of the splenic rat lymphocytes (counts per minutes, the tritium-labelled uridine incorporation test); (a) 7 days after therapy withdrawal; (b) 14 days after therapy withdrawal. Legend: The treatment of animal groups: 1;2;3—hydrophilic MTX-loaded liposomes (0.2;0.3;0.4 mg/kg b.w.); 4;5;6—hydrophobic MTX-loaded liposome (0.2;0.3;0.4 mg/kg b.w.); 7;8;10—MTX solution for injection (0.2;0.3;0.4 mg/kg b.w.); 9—control (liposomes, 0.1 mL/100 g/kg b.w.).

stimuli, but the effect is not persistent. Only the MTX-loaded liposomes exert an immunosuppressive action by limiting the *ex vivo* response of splenocytes to ConA. In this case, an anergic state of splenocytes seems to be triggered. Fourteen days after treatment discontinuation, all investigated MTX formulations inhibit splenocytes response to ConA. Accordingly, MTX liposomes exert a lasting antiproliferative action at critical doses. It is worth noticing that low and high doses of hydrosoluble MTX liposomes induce splenocytes anergy, namely activation of resting cells and reduced responses *ex vivo* to Con A. Hydrophobic MTX-loaded liposomes seem to be most efficacious in restraining splenocytes activation.

The results indicate that MTX loaded in liposomes has a more evident impact on the immune status than MTX solution for injection, and hydrophobic MTX incorporated in liposomes seems to be active at the lowest doses (**Figure 18**).

The evaluation of the haematological and biochemical parameters indicates a low toxic effect of MTX in arthritic rats in the applied treatment regimen. Erythrocyte count was not significantly affected and between erythrocyte parameters series good correlation (correlation coefficients of >0.90) was found. Transaminases activities were weak and irregularly affected, registering slight increases (especially AST) at highest MTX doses 7 days after the last administration. The creatinine and urea serum levels were not significantly affected [32].

MTX treatment induced discrete to moderate and reversible histopathological changes in the liver and the kidney. However, a more pronounced impairment in the kidney (glomerular stasis and the increase of the vascular network volume, as a result of circulation disturbances, as well as tubular nephritis and medullary mononuclear cell infiltration), depending on the type of treatment (MTX liposomes or MTX solution for injection) and of the administered dose [46], has been noticed.

6. Conclusion: key results

Several types of poly-disperse liposomal systems containing both hydrosoluble methotrexate and hydrophobic methotrexate were prepared by two methods: lipid film hydration and reverse-phase evaporation. The last one was selected due to the shorter working time and the higher encapsulation efficiency. The liposomal poly-dispersion was extruded to obtain a liposomal monodispersion. MTX liposomes were characterized by VEM, TEM and DLS. The obtained liposomes had the diameters of microns size. The untrapped drug was removed and the concentration of entrapped MTX was chromatographically determined. The encapsulation efficiency was satisfactory and similar for PC:MTX (10:1) liposomes and for PC:CH:MTX (10:1:1) liposomes. The presence of CH in liposomal membrane increases the rigidity and the hydrophobicity of the membrane. A higher hydrophobic character of liposomal membrane means a larger loading efficiency of hydrophobic MTX.

Hydrophobic MTX loaded in liposomes tends to restrain the tumoural multiplication of K562 cells and clearly inhibits RNA synthesis, suggesting that activation events are primarily the target of the drug, and not the neoplastic proliferation of lymphoblasts.

The methotrexate liposomes exhibited significant anti-inflammatory activity and showed reduced toxicity. Given that the encapsulating of the drug in vector systems may result in the increasing concentration at the site of action, the liposomes with methotrexate represent a targeted therapy with an optimized therapeutic efficacy—risk toxicity ratio.

Acknowledgments

We thank Manuela Florea-Spiroiu, PhD Lecturer, for assistance with liposomes preparation.

Author details

Anne Marie Ciobanu¹, Maria Bârcă², Gina Manda², George Traian Alexandru Burcea Dragomiroiu¹ and Daniela Luiza Baconi^{3*}

*Address all correspondence to: daniela_baconi@yahoo.com

1 Drug Control Department, Faculty of Pharmacy, “Carol Davila” University of Medicine and Pharmacy, Bucharest, Romania

2 Radiobiology Laboratory, “Victor Babes” National Institute of Pathology, Bucharest, Romania

3 Toxicology Department, Faculty of Pharmacy, “Carol Davila” University of Medicine and Pharmacy, Faculty of Pharmacy, Bucharest, Romania

References

- [1] Abraham SA, Waterhouse DN, Mayer LD, Cullis PR, Madden TD, Bally MB. The liposomal formulation of doxorubicin. *Methods in Enzymology*. 2005;**391**:71-97. DOI: 10.1016/S0076-6879(05)91004-5
- [2] Pentak D, Kozik V, Bąk A, Dybał P, Sochanik A, Jampilek J. Methotrexate and cytarabine-loaded nanocarriers for multidrug cancer therapy. *Spectroscopic Study Molecules*. 2016;**21**(12):pii:E1689. DOI: 10.3390/molecules21121689
- [3] Popovici A, Antofi N. *Lipozomi ca vectori medicamentosi* (available in Romanian language). Cluj Napoca: Romsver; 2004
- [4] Hong MS, Lim SJ, Oh YK, Kim CK. pH-sensitive, serum-stable and long-circulating liposomes as a new drug delivery system. *Journal of Pharmacy and Pharmacology*. 2002;**54**(1):51-58
- [5] Groll AH, Mickiene D, Piscitelli SC, Walsh TJ. Distribution of lipid formulations of amphotericin B into bone marrow and fat tissue in rabbits. *Antimicrobial Agents and Chemotherapy*. 2000;**44**(2):408-410

- [6] Groll AH, Mickiene D, Werner K, Petraitiene R, Petraitis V, Calendario M, Field-Ridley A, Crisp J, Piscitelli SC, Walsh TJ. Compartmental pharmacokinetics and tissue distribution of multilamellar liposomal nystatin in rabbits. *Antimicrobial Agents and Chemotherapy*. 2000;**44**(4):950-957
- [7] Lim HJ, Masin D, Madden TD, Bally MB. Influence of drug release characteristics on the therapeutic activity of liposomal mitoxantrone. *Journal of Pharmacology and Experimental Therapeutics*. 1997;**281**(1):566-573
- [8] Krishna R, Ghiu G, Mayer LD. Visualization of bioavailable liposomal doxorubicin using a non-perturbing confocal imaging technique. *Histology and Histopathology*. 2001;**16**(3):693-699
- [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. *Experimental Biology and Medicine*. 2001;**226**(6):559-564
- [10] Konigsberg PJ, Debrick JE, Pawlowski TJ, Staerz UD. Liposome encapsulated aurothiomalate reduces collagen-induced arthritis in DBA/1J mice. *Biochimica et Biophysica Acta*. 1999;**1421**:149-162
- [11] Akbarzadeh A, Rezaei-Sadabady R, Davaran S, Joo SW, Zarghami N, Hanifehpour Y, Samiei M, Kouhi M, Nejati-Koshki K. Liposome: Classification, preparation, and applications. *Nanoscale Research Letters*. 2013;**8**:102. DOI: 10.1186/1556-276X-8-102
- [12] Bangham AD, Standish MM, Watkins JC. Diffusion of univalent ions across the lamellae of swollen phospholipids. *Journal of Molecular Biology*. 1965;**13**:238-252
- [13] Chang H, Yeh MK. Clinical development of liposome-based drugs: Formulation, characterization, and therapeutic efficacy. *International Journal of Nanomedicine*. 2012;**7**:49-60
- [14] Genestier L, Paillot R, Fournel S, Ferraro C, Miossec P, Revillard JP. Immunosuppressive properties of methotrexate: Apoptosis and clonal deletion of activated peripheral T cells. *Journal of Clinical Investigation*. 1998;**102**(2):322-328. DOI: 10.1172/JCI2676
- [15] Pachner AR, Amemiya K, Delaney E, O'Neill T, Hughes CA, Zhang WF. Interleukin-6 is expressed at high levels in the CNS in Lyme neuroborreliosis. *Neurology*. 1997;**49**(1):147-152
- [16] Cutolo M, Sulli A, Pizzorni C, Seriolo B, Straub RH. Anti-inflammatory mechanisms of methotrexate in rheumatoid arthritis. *Annals in Rheumatoid Diseases*. 2001;**60**(8):729-735. DOI: 10.1136/ard.60.8.729
- [17] Weinblatt ME, Weissman BN, Holdsworth DE, Fraser PA, Maier AL, Falchuk KR, Coblyn JS. Long-term prospective study of methotrexate in the treatment of rheumatoid arthritis: 84-month update. *Arthritis & Rheumatology*. 1992;**35**(2):129-137
- [18] Maetzel A, Bombardier C, Strand V, Tugwell P, Wells G. How Canadian and US rheumatologists treat moderate or aggressive rheumatoid arthritis: A survey. *Journal of Rheumatology*. 1998;**25**:2331-2338

- [19] Krause D, Schleusser B, Herborn G, Rau R. Response to methotrexate treatment is associated with reduced mortality in patients with severe rheumatoid arthritis. *Arthritis & Rheumatology*. 2000;**43**(1):14-21. DOI: 10.1002/1529-0131(200001)43:1<14::AID-ANR3>3.0.CO;2-7
- [20] Kapoor B, Singh SK, Gulati M, Gupta R, Vaidya Y. Application of liposomes in treatment of rheumatoid arthritis: Quo vadis. *The Scientific World Journal*. 2014;**2014**(ID978351):1-17. DOI: 10.1155/2014/978351
- [21] Bader RA. The development of targeted drug delivery systems for rheumatoid arthritis treatment. In: Lemmey AB, editor. *Rheumatoid Arthritis – Treatment*. InTech; 2012. pp. 111-132. ISBN 978-953-307-850-2 <https://www.intechopen.com/books/rheumatoid-arthritis-treatment/the-development-of-targeted-drug-delivery-systems-for-rheumatoid-arthritis-treatment>
- [22] Cintează O. *Chimie fizică a medicamentului* (available in Romanian language). Bucharest: Ars Docenti; 2002
- [23] Bârcă M, Bălălaşu D, Ciobanu AM, Olteanu M, Dudău M, Cintează O. Preparation and characterization of methotrexate-loaded liposomes. *“Ovidius” University Annals of Medical Science – Pharmacy*. 2004;**II**(I):137-142
- [24] Kono K, Henmi A, Takagishi T. Temperature-controlled interaction of thermosensitive polymer-modified cationic liposomes with negatively charged phospholipid membranes. *Biochimica et Biophysica Acta*. 1999;**1421**(1):183-197. DOI: 10.1016/S0005-2736(99)00123-6
- [25] Szoka F Jr, Papahadjopoulos D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse/phase evaporation. *Proceedings of the National Academy of Sciences United States of America*. 1978;**75**(9):4194-4196
- [26] Szoka F Jr, Papahadjopoulos D. Comparative properties and methods of preparation of lipid vesicles (liposomes). *Annual Review of Biophysics and Bioengineering*. 1980;**9**:467-508. DOI: 10.1146/annurev.bb.09.060180.002343
- [27] Paternostre M, Ollivon M, Bolard J. Liposomes: Preparation and Membrane Protein Reconstitution. In: Prasad R, editor. *Manual on Membrane Lipids*. Springer Lab Manual; 1996. pp. 202-247 ISBN: 978-3-642-48970-9 (Print) 978-3-642-79837-5 (Online)
- [28] Nenitescu CD. *Chimie organică, vol 1* (available in Romanian language). Bucharest: Editura Pedagogică; 1980
- [29] Lee KD, Nir S, Papahadjopoulos D. Quantitative analysis of liposome-cell interactions in vitro: Rate constants of binding and endocytosis with suspension and adherent J774 cells and human monocytes. *Biochemistry*. 1993;**32**(3):889-899. DOI: 10.1021/bi00054a021
- [30] Miller CR, Bondurant B, McLean SD, McGovern KA, O'Brien DF. Liposome-cell interactions in vitro: Effect of liposomes surface charge on the binding and endocytosis of conventional and sterically stabilized liposomes. *Biochemistry*. 1998;**37**(37):12875-12883. DOI: 10.1021/bi980096y

- [31] Pleyer U, Grammer J, Kosmidis P, Ruckert DG. Analysis of interactions between the corneal epithelium and liposomes: Qualitative and quantitative fluorescence studies of a corneal epithelial cell line. *Survey of Ophthalmology*. 1995;**39**(1):S3-S16
- [32] Bârcă M, Baconi DL, Ciobanu AM, Burcea GTA, Bălălaşu C. 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. *Farmacia*. 2013;**61**(1):220-228
- [33] Firestein GS. Novel therapeutic strategies involving animals, arthritis, and apoptosis. *Current Opinion in Rheumatology*. 1998;**10**(3):236-241
- [34] Youssef PP, Smeets TJ, Bresnihan B, Cunnane G, Fitzgerald O, Breedveld F, Tak PP. Microscopic measurement of cellular infiltration in the rheumatoid arthritis synovial membrane: A comparison of semiquantitative and quantitative analysis. *British Journal of Rheumatology*. 1998;**37**(9):1003-1007
- [35] Goronzy JJ, Weyand CM. T and B cell-dependent pathways in rheumatoid arthritis. *Current Opinion in Rheumatology*. 1995;**7**(3):214-221
- [36] Goronzy JJ, Weyand CM. T cells in rheumatoid arthritis. Paradigms and facts. *Rheumatic Disease Clinics of North America*. 1995;**21**(3):655-674
- [37] Blä S, Engel JM, Buemester GR. The immunologic homunculus in rheumatoid arthritis. *Arthritis & Rheumatology*. 1999;**42**:2499-2506
- [38] Ismail AA, Snowden N. Autoantibodies and specific serum proteins in the diagnosis of rheumatological disorders. *Annals in Clinical Biochemistry*. 1999;**36**(5):565-578
- [39] Bayraktar A, Hudson S, Watson A, Fraser S. Arthritis. *Pharmaceutical Journal*. 2000;**264**(7078):57-68
- [40] Wessels JAM, Huizinga TWJ, Guchelaar H-J. Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis. *Rheumatology*. 2008;**47**:249-255
- [41] Cronstein B. How does methotrexate suppress inflammation? *Clinical and Experimental Rheumatology*. 2010;**28**(5 Suppl 61):S21-S23
- [42] van Ede AE, Laan RF, Blom HJ, De Abreu RA, van de Putte LB. Methotrexate in rheumatoid arthritis: An update with focus on mechanisms involved in toxicity. *Seminars in Arthritis Rheumatology*. 1998;**27**(5):277-292
- [43] O'Dell JR. Methotrexate use in rheumatoid arthritis. *Rheumatic Disease Clinics of North America*. 1997;**23**(4):779-796
- [44] Liang LS, Jackson J, Min W, Risovic V, Wasan KM, Burt HM. Methotrexate loaded poly(L-lactic acid) microspheres for intra-articular delivery of methotrexate to the joint. *Journal of Pharmaceutical Science*. 2004;**93**(4):943-956
- [45] Theisen-Popp P, Muller-Peddinghaus R. Antirheumatic drug profiles evaluated in the adjuvant arthritis of rats by multiparameter analysis. *Agents and Actions*. 1994;**42**:50-55

- [46] Bârcă M, Baconi DL, Ciobanu AM, Militaru M, Burcea GTA, Bălălaşu C. Comparative evaluation of methotrexate toxicity as solution for injection and liposomes following a short-term treatment in a murine model of arthritis: Note II. Histopathological changes. *Farmacia*. 2013b;**61**(5):939-947
- [47] Randall LO, Selitto JJ. A method for measurement of analgesic activity on inflamed tissue. *Archives in International Pharmacodynamics Therapy*. 1957;**111**:409-419
- [48] 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
- [49] Gottschalk O, Metz P, Dao Trong ML, Altenberger S, Jansson V, Mutschler W, Schmitt-Sody M. Therapeutic effect of methotrexate encapsulated in cationic liposomes (EndoMTX) in comparison to free methotrexate in an antigen-induced arthritis study in vivo. *Scandinavian Journal of Rheumatology*. 2015;**44**(6):456-463
- [50] Bârcă M, Ciobanu AM, Bălălaşu D, Baconi D, Ilie M, Neagu M, Manda G. Short-term treatment with methotrexate-loaded liposomes in a murine model of arthritis. *Toxicology Letters*. 2005;**158**(Suppl 1):S91-S92