Methotrexate transport mechanisms: the basis for targeted drug delivery and β-folate-receptor-specific treatment

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ABSTRACT

Methotrexate (MTX) plays a pivotal role in the treatment of rheumatoid arthritis (RA). The transport mechanisms with which MTX reaches is target after application are an important part of MTX pharmacology and its concentration in target tissue such as RA synovial membrane might strongly influence the effectiveness of the drug. Physiological plasma protein binding of MTX to albumin is important for the distribution of MTX in the body and relative high concentrations of the drug are found in the liver. However, targeted drug delivery into inflamed joints and increased anti-arthritic efficiency can be obtained by covalent coupling of MTX ex-vivo to human serum albumin (MTX-HSA) or in-vivo *to endogenous albumin mediated through the MTX-pro-drug AWO54. High expression of the folate receptor β (FR-β) on synovial macrophages of RA patients and its capacity to mediate binding and uptake of MTX has been demonstrated. To further improve drug treatment of RA, FR-β specific drugs have been developed and were characterised for their therapeutic potency in synovial inflammation. Therefore, different approaches to improve folate inhibitory and FR-β specific therapy of RA beyond MTX are in development and will be described.*

Introduction

The therapeutic effect of methotrexate (MTX) in rheumatic diseases mainly depends on its potential to be active as an anti-inflammatory agent in target tissue such as synovial pannus of rheumatoid arthritis (RA). Therefore, the transport mechanisms by which MTX reaches its targets and is transported into the cell are a critical component of MTX efficiency in the treatment of RA.

MTX blood transport and organ distribution

MTX is a small hydrophilic molecule with a molecular weight of 454 and a chemical structure related to folic acid. After either intravenous, subcutaneous or intramuscular injection, it rapidly reaches a peak concentration in plasma after about 1.3h (1). When administered orally, it is absorbed in the proximal jejunum with only slightly slower kinetics, reaching peak plasma levels 1.5h after intake of the drug (2). Once in the plasma, the transport mechanism of protein binding becomes active, which is almost completely mediated by the plasma albumin. MTX has a mean rate of albumin binding of 42–57% (2), although in some studies a range of protein binding between 20% and 70% has been reported (3, 4). In contrast, 7OH-MTX is over 90% bound to plasma albumin in the circulation. This metabolite of MTX produced by aldehyde oxidases through hepatic metabolisation has a reduced anti-folate activity in comparison to native MTX (2). Because of the intermediate rate of albumin binding, displacement of MTX by other protein-bound drugs such as NSAIDs has only limited effects on the concentrations of free MTX and is not clinically relevant in low-dose MTX treatment as used for rheumatic diseases.

However, the binding of MTX to albumin has consequences on the distribution of MTX in the body and its therapeutic effects and toxicity. Albumin is a ubiquitous protein that constitutes 50-70% of the pool of plasma proteins. It has a particularly high rate of extravasations, with about 6% of the entire albumin leaving the intravascular compartment every hour, mainly by means of the mechanism of transcytosis through the endothelial cell layer (5, 6). In contrast, the extravasations rate of immunoglobulins is only about 3%. Human albumin has a biological half-life of 19 days. Statistically, every single molecule of albumin passes through the circulation about 15,000 times and through the endothelium about 15 times, and then returns to the intravascular

compartment (5, 6). Drugs that are bound to albumin by plasma protein binding are passengers on this journey, for at least part of the way. Plasma protein binding of drugs to albumin is not based on covalent binding but rather on reversible, "adhesive" binding (6, 7). This is based on interaction between hydrophobic and lipophilic parts of the albumin and ligand molecules as well as negatively and positively charged binding sites. Albumin has six known binding areas for organic and inorganic molecules, such as hormones, vitamins and fatty acids, as well as drugs, one of which is MTX. When an albumin-ligand complex passes through the capillary bed of an organ such as the liver, it may extravasate into the tissue. When it comes into contact with organ cells, the albumin-drug complex is reversibly bound to the surface of the cells. This leads to the dissolution of the protein-ligand complex, and the ligand is taken up by the cell. In contrast, the albumin molecule mostly remains extracellular and is slowly transported through the interstitium to finally reach the circulation again by lymphatic transport.

This mechanism has been extensively studied for the binding of complexes of long-chain fatty acids with albumin to hepatocytes (8). These are bound to albumin at the site of a hydrophobic channel in the albumin molecule and as well with ionic binding at the outside of the molecule. This binding induces a conformational change of the albumin molecule. When the complex comes into contact with surfaces such as the cell membrane of hepatocytes, a further conformational change results in increased binding capacity of albumin to this surface and finally the release of the ligand molecule (9). It has been calculated that with every passage through the liver, 25% of the albumin-bound drugs are released and therefore prepared to be taken up intracellularly in this way (6). Initially, it was speculated that a specific receptor for albumin on hepatocytes mediates this interaction (8). However, it could be shown that the surface effect is not cell specific and can occur with other cell types and even non-biologic surfaces (9).

A single-pass uptake of fatty acids in the liver by albumin transport has a physiologic role to ensure rapid metabolisation of these molecules. Moreover, the transport of potentially toxic substances by albumin is important for their rapid elimination before they can reach and damage other organs. However, in the case of MTX and other albumin-bound drugs, the mechanism of albumin transport and surface-induced dissolution of the complex may favour accumulation in the liver and other organs such as the kidney. Some results that support this hypothesis will be presented below. An exception is the central nervous system, in which albumin extravasation is prevented by the blood-brain barrier. Other tissues such as musculature, skin or bone, have not been studied in detail. The non-uniform distribution of albumin-bound drugs may therefore have consequences for the relative exposure of target tissue to MTX and the rate of organ toxicity.

We used the murine collagen-induced arthritis model (CIA) to study MTX pharmacokinetics (10, 11). One hour after intravenous application of radioactive-labelled MTX (^{3}H-MTX) at a dose of 0.03µg/kg body weight, a maximum of 32% of the administered label accumulated in the liver, a rate which slowly decreased to 18% after 48h. In contrast, in each inflamed hind paw of the arthritic mice, a maximum of 0.5% of the applied ^{3}H-MTX was detected 1h after intravenous application. This rate slowly decreased to 0.25% and then remained stable over the next 48h (10). In comparison, each inflamed hind paw contributes about 0.75% to total body weight. The uptake of ^{3}H-MTX per mg tissue in inflamed paws, liver, kidney and blood over 24h is shown in Figure 1. It is remarkable that the low-dose MTX treatment in this murine model resulted in a 19-fold higher uptake of MTX in the liver and a 15-fold in the kidney per mg of tissue in comparison to the inflamed paws 1h after application (Table I). However, in these experiments, the entire inflamed paws were measured, rather than the synovial membrane of these joints alone (10, 11). This finding corresponds to other studies in which tropism of MTX to the liver have been shown before. For example, Bischoff *et al*. estimated a ratio of 3:1 of MTX concentration between liver tissue and plasma in a mathematical model; however high plasma concentrations of MTX as seen in cancer chemotherapy were assumed (12). Kremer *et al*. detected MTX in liver biopsy specimens of RA patients predominantly in the polyglutamated form (13), but a comparison to the amounts of MTX in synovial samples was not performed.

Although large parenchymatous organs such as the liver are most likely

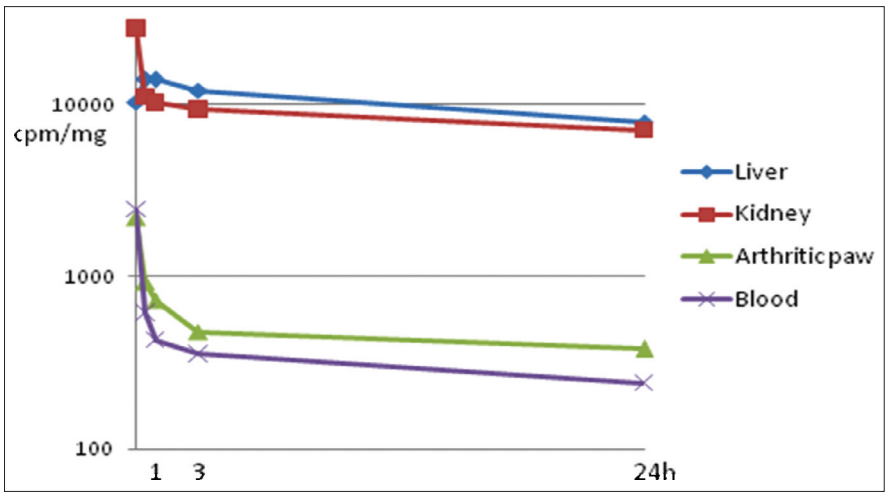

Fig. 1. Relative uptake of ^{3}H-MTX in different organs of mice with CIA arthritis. Three mice each were sacrificed 5min, 30min, 1h, 3h and 24h after IV application of 0.03µg/kg ^{3}H-MTX. Counts per minute (cpm) were measured with liquid scintillation counting and cpm per mg tissue was calculated. Only paws with arthritis grade 3 were used. Standard deviation (not shown) was <5% of total cpm in all cases. The curves show the high relative uptake of MTX in liver and kidney in this model when given in low doses. Data are based on previously unpublished analyses from (10, 11).

Table I. MTX blood transport and organ distribution of MTX and albumin-coupled MTX. Pharmacokinetic studies were performed with radioactively labelled drugs, either ^{3}H-MTX (0.03µg/kg) for studying MTX or ^{111}In-DTPA-HSA (400µg per mouse = ~16μg/kg) for the study of albumin conjugates. Data are partially based on previously unpublished analyses from (10, 11).

	MTX	Albumin-coupled MTX
General characteristics		
Albumin binding	Reversible, "adhesive" plasma protein binding (42–57%)	Covalent coupling *ex vivo* (MTX-HSA) or through a peptide spacer to endogenous albumin *in vivo* (AWO54)
Release of the free drug from albumin binding	Release of the ligand by surface-induced conformational change of the albumin molecule in parenchymatous organs	Cleaving of the prodrug by intralysosomal uptake and metabolisation of albumin or alternatively extracellular proteinases (shown only for AWO54)
Pharmacokinetics in the CIA model 1 h after IV injection		
Percent uptake per arthritic hind paw	0.5%	2%
Increase of uptake in arthritic *vs*. non-arthritic paws	2–4 fold	6–8 fold
Relative uptake per mg tissue liver/arthritic paw	19:1	1:1
Relative uptake per mg tissue kidney/arthritic paw	15:1	1.5:1

the main site of the drug dissolution from reversible albumin binding, this transport also targets the human synovium, as shown for NSAIDs which are carried by albumin to the articular site and are detectable in synovial fluid (14). Moreover, experimentally induced subcutaneous inflammation accumulates Evans blue, a pigment with high affinity for binding to albumin in the inflammatory tissue by the mechanism of albumin transport (15). While the distribution of MTX after low-dose treatment in RA patients has not been investigated extensively to date, some studies have shown that at least some accumulation in arthritic joints takes place: Measurements of synovial and bone tissue of RA patients who had undergone surgical procedures showed about 10-fold higher concentrations of MTX in the synovial tissue than in the plasma 20h after application. Tishler *et al*. found about 3-fold higher concentrations of MTX in synovial fluid of patients with RA in comparison to plasma levels 24h after application (16). This shows a retention effect of MTX in the inflamed joints; however, liver and kidney concentrations – at potential sites of dose limiting toxicity – were not investigated (17).

In conclusion, the specific pharmacologic properties of MTX may result in a relatively high uptake of the drug in liver and kidney compared to those in arthritic joints in the murine CIA model. It is likely that this is partly due to plasma protein binding of the drug to albumin and the specific properties of albumin to release its ligand by contact with cell surfaces during passage through these organs. However, for methodological reasons, the animal studies described above were performed with very low doses of radioactively labelled MTX of only 0.03µg/kg, which may have influenced the distribution of MTX (12). Some publications point towards an accumulation of MTX in the inflamed joints as well, but data of patients with RA comparing synovial and organ uptake of MTX after low-dose treatment are not available.

Targeted drug delivery by covalent coupling of MTX to albumin

The unfavourable pharmacokinetic properties of MTX can be overcome when MTX is covalently coupled to a carrier targeting the molecule to the site of inflammation. Albumin can be used as this type of carrier if it is covalently coupled to MTX to prevent dissolution of the complex during organ passage. While albumin is produced by the liver, only a relatively small proportion is metabolised there. In healthy subjects, the main metabolisers of albumin are skin fibroblasts and musculature (5, 6). However, in tumour patients or patients with severe arthritis, the malignant or inflammatory tissue metabolises albumin in large amounts to satisfy energy and nitrogen demand. Synovial tissue of patients with RA has been shown to have an approximately 6-fold increase in albumin extravasation as compared to non-inflamed synovial tissue (18) and the tissue has a high albumin turnover due its hypermetabolic state (19-21).

We recently introduced albumin-coupled MTX (MTX-HSA) as a novel conjugate, which consists of human serum albumin (HSA) from blood donors and MTX covalently coupled to each other at a 1:1 molecular ratio (10, 22-24). MTX-HSA has a substantially prolonged half-life in the circulation and due to the increased extravasation of albumin into inflamed tissue and metabolisation of albumin by cells with a high protein turnover, it accumulates in inflamed joints of mice with collagen-induced arthritis (CIA) (10). We demonstrated that in comparison to equivalent concentrations of MTX, MTX-HSA is significantly more effective than MTX in the prevention of arthritis in CIA mice (10) and acts synergistically to MTX in this model (22). Moreover, this novel therapeutic approach of albumin-based targeted drug delivery was tested *in vitro* in human RA-SF as well as in peripheral blood mononuclear cells of RA patients which both metabolise albumin conjugates and accumulate them intracellularly (10;22). In a human model of RA using mice with severe combined immunodeficiency (SCID) co-transplanted with human cartilage and SF from patients with RA (25), synovial fibroblast invasion and cartilage degradation was reduced by MTX-HSA as well as by MTX *in vivo* (23). Albumin conjugates have a more favourable distribution for

the treatment of arthritis than MTX. We showed that the uptake of albumin conjugates in arthritic paws of CIA mice is increased more extensively in comparison to non-arthritic paws than MTX (10). Moreover, the ratio between uptake in the liver and kidneys, on the one hand, and in the inflamed paws, on the other, is better in comparison to MTX. These results, which are based on previously unpublished analyses of our data from (10) and (11), are summarised in Table I.

The use of HSA from blood donors is subject to legal restrictions. For this reason, in order to avoid using HSA, we aimed to find a drug that couples to endogenous albumin after application. In recent publications, Kratz *et al*. demonstrated that it is possible to synthesise prodrugs which are bound to the cysteine-34 position of endogenous albumin after systemic application (26-28). Recently, we demonstrated that the albumin-binding prodrug of methotrexate AWO54 with the formula EMC-D-Ala-Phe-Lys-Lys-MTX specifically binds to the cysteine-34 position (29). Similar to exogenous albumin-drug conjugates such as MTX-HSA, this endogenous complex is transported to the joint inflammation. The peptide spacer that is chemically bound to the MTX molecule, can be cleaved by proteases such as cathepsin B and plasmin at the site of inflammation, leaving albumin and MTX-lysine. We showed that after intravenous injection of AWO54, a superior therapeutic effect in comparison to MTX in the treatment of CIA mice was achieved. We therefore concluded that MTX-lysine is an active drug which is targeted to inflammation by coupling to albumin. To obtain a similar effect, only about 20% of the MTX-equivalent dose of AWO54 had to be given in comparison to free MTX. The efficacy of the drug was tested in different stages of CIA in the mice: while MTX lost its efficacy in later stages of the disease, AWO54 remained active in reducing arthritis even in later stages (29).

In conclusion, albumin, when covalently coupled to a drug at a 1:1 ratio, is a promising drug carrier to carry MTX to the site of synovial inflammation in RA and has the potential to increase therapeutic efficiency and reduce accumulation in liver and kidney. This prodrug, either as a ligand for binding to endogenous albumin (AWO54) or coupled to HSA (MTX-HSA) is accumulated at the site of inflammation. In contrast to the physiologic plasma protein binding of MTX to albumin, MTX-HSA or AWO54 release MTX (or MTX-lysine) as an active drug, either when albumin is taken up intracellularly to be metabolised in the lysosome compartment or in the presence of extracellular proteinases such as cathepsin B or plasmin (AWO54) but not during regular organ passage. With this novel approach, a higher specificity for inflamed tissue and a better therapeutic response to RA may be achieved. Clinical studies for use in the treatment of RA are in preparation.

Table II. Folate-transport mechanisms and their cellular distribution and specificity for folate and MTX.

Folate transport mechanisms	Major cellular expression	Utilisation by folate and MTX
Non-receptor dependent		
Reduced folate carrier (RFC)	Ubiquitous distribution	MTX, 5-formyltetrahydrofolate (leucovorin), Folate
Receptor dependent		
Folate receptor-α (FR-α)	Epithelial cells (luminal surface), malignant cells	Folate
Folate receptor-β (FR-β)	Differentiated myelomonocytic cells, in particular activated monocytes/ macrophages, synoviocytes, neutrophils	Folate and MTX (assumed glycosylation in activated cells)
Folate receptor-γ (FR-γ)	Secreted receptor form, constitutively expressed in hematopoietic tissue	Folate

Cellular uptake of MTX in target tissue

When MTX reaches the target tissue such as synovial pannus in RA, it has to be taken up intracellularly to become effective as an anti-inflammatory agent. The cellular uptake in particular in the different target cell populations is therefore a further critical step in the balance between effect and potential side effect of the drug.

There are several transport mechanisms by which folate or its inhibitors can enter the cell (Table II). MTX has a particularly high affinity to the reduced folate carrier (RFC), a transmembrane folate transport mechanism that has a ubiquitous distribution in the body and is constitutively expressed. In contrast, there are the folate receptors (FR) -α,-β and -γ, which, in non-activated states, have only very low affinity for MTX (30). FR-β is a differentiation marker of the myelomonocytic lineage of hematopoietic cells and is also highly expressed on mature neutrophil granulocytes. FR-α is mainly expressed on epithelial cells and malignant tumours and FR-γ is the secreted form of the receptor (Table II). It has been shown that activated synovial macrophages from patients with RA selectively express FR-β and that MTX is transported through this receptor in these cells (31). As FR-β on non-activated cells has only a low binding affinity for folates, it was speculated that in activated states, glycosylation of FR-β on macrophages might occur and that this may result in increased folate, as well as MTX-binding capacity of the receptors (30). Immunohistochemistry and PCR analysis of synovial tissue samples of RA patients confirmed that FR-β expression is a phenomenon highly specific to macrophages in the synovial membrane of RA patients but not in non-inflammatory synovial tissue. In contrast, T-lymphocyte areas in the synovium show no FR-β expression (32). Due to these properties, FR-β has been recognised as an attractive target for imaging of arthritis (33).

The specific up-regulated and glycosylated FR-β on synovial macrophages may be an anchor for folate antagonists, such as MTX, at the site of joint

inflammation. However, the affinity of MTX to RFC in particular, resulting in widespread uptake, limits the magnitude of an inflammation-specific effect of FR-β utilisation of MTX in RA. Therefore, anti-folate drugs that in contrast to MTX, would be FR-β specific would have a higher effect on synovial macrophages and lower effect on other cell types that take up MTX by the ubiquitously expressed RFC. A higher therapeutic effect and a lower rate of side effects by FR-β-specific anti-folates in comparison to MTX may possibly be the result.

New approaches to β-folate receptor specific treatment

Several experimental therapeutic concepts using the relative specificity of FR-β for synovial inflammation in RA have been developed. Nagayoshi *et al*. presented an approach in which an anti-FR-β antibody crosslinked with the 38-kd portion of pseudomonas exotoxin A (PE38) was tested. They showed that this conjugate is able to induce apoptosis in RA-synovial macrophages and inhibit TNF-α production *in vitro* (34). This approach was modified by coupling a recombinant variable-region antibody fragment (Fv) against FR-β to PE38 (35). Treatment with this conjugate reduced the number of macrophages, activated fibroblast-like cells, endothelial cells and proliferating cells and increased the number of apoptotic cells in the synovial implant of a human SCID-mouse model for RA *in vivo*. The authors propose the further development of Fv-PE38 for intra-articular treatment of arthritis (35).

Van der Heijde *et al*. recently presented a very different approach to folate receptor-specific treatment (32, 36). They characterised new folate antagonistic drugs for their capacity to selectively utilise FR-β. Second generation anti-folates were developed for the treatment of patients with malignant diseases, mostly from the perspective of circumventing common mechanisms of resistance to MTX, including impaired transport via the RFC. These new anti-folates have a broad range of different properties concerning their use of the RFC transport mechanism, their binding capacity of FR as well as the targeting of different key enzymes of folate metabolism. Van der Heijde *et al*. examined these novel drugs in terms of properties that most likely will be beneficial for the treatment of synovial inflammation. Usage of the RFC system, due to its non-specific expression is a non-preferred property, was tested by measuring the growth inhibition of a non-FR-expressing human monocytic-macrophage cell line THP-1. Two out of ten tested new folate inhibitors (BGC945 and CB300635) were shown not to be active in this RFC-specific system. When the relative binding affinities of the anti-folates to FR-α and -β were tested using the FR-α expressing cell line KB and FR-β-transfected CHO cells (CHO/FR-β), the two latter drugs showed strong binding to both receptors comparable to those of folate. In contrast, the binding of MTX was about 50-fold lower. Specificity for one of the FR subtypes α or β was not detectable in either of the drugs. Finally, the growth inhibition of CHO/FR-β by BGC945 and CB300635 in contrast to wild-type CHO cells was examined. It appeared that only BGC945 had the property to inhibit growth of CHO/FR-β cells in low concentrations and this was completely inhibited by the addition of folate, demonstrating the receptor specificity of the process.

In summary, the incremental approach (32) resulted in the identification of the novel folate antagonist BGC945, which is active through FR but has only a little affinity to the RFC. Due to the abundant FR-β expression on activated macrophages in synovial tissue, this new drug therefore is a candidate drug for a novel approach to selective therapeutic intervention that targets FR-β and is a promising candidate drug for further preclinical and clinical studies for the treatment of RA. However, it obviously remains to be seen whether the targeting to FR-β will deliver on these promises. FR-β is highly expressed on synovial macrophages and most likely on neutrophils as well. Other cell types that are also thought to play an important role in the pathology of RA are not targeted with this approach. This is in contrast to MTX, which increases the rate of apoptosis of T-lymphocytes in RA (37) and also reduces cartilage invasion and degradation in a human SCID-mouse model for RA, which is exclusively mediated through activated synovial fibroblasts (23). An FR-β-specific folate antagonist, in contrast to MTX, therefore may miss these cell types. Whether or not this reduces its therapeutic effectiveness remains to be seen. Finally, an FR-specific approach of folate antagonistic drugs uses the same transport system as folate itself. As a result, the drug competes with folate. Therefore, its therapeutic action may be influenced by folate intake, making the efficiency of this treatment sensitive to *e.g*. the uncontrolled intake of folate-containing nutritional additives. However, despite these questions, which will remain unanswered until the clinical development of the drug, the FR-ß-specific approach represents a promising novel approach for folic acid-antagonist drug treatment in RA.

In conclusion, MTX is a drug with an exceptional potency to treat inflammatory joint disease and in particular synovial inflammation in patients with RA. The important role played by MTX in the treatment of this disease is achieved despite some unfavourable pharmacokinetic properties of the substance. This includes an only slow intracellular accumulation of polyglutamated MTX, which relays the initial effect of the drug and relative tropism to the liver and kidney that contributes to dose limiting toxicity. Moreover, the mechanisms for the intracellular uptake of the drug are non-tissue-specific and a range of folate transporters with some cell specificity are not differentially targeted by MTX. A new approach uses targeted drug delivery by covalent coupling to albumin either *ex vivo* with human serum albumin or in vivo by coupling of a prodrug to endogenous albumin. This may improve the exposure of synovitis to MTX and result in better treatment responses. Drugs that specifically bind to the FR-β constitute an additional promising approach, as FR-β has been shown to have a relative specificity for synovial macrophages. Drug compounds that specifically deplete those cells from

synovium by targeting FR-β are in development. Moreover, FR-β-specific folate inhibitors will be more effector-cell specific than MTX and therefore may allow further optimisation of the successful principle of folate inhibition in the treatment of RA in future.

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