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Differential Docking of Methotrexate and Metabolite on Renal Transporters and Dihydrofolate Reductase

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KEYWORDS

Methotrexate

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ABSTRACT:

Introduction: Methotrexate is widely used for cancer and autoimmune disease therapy, but its use is often limited by nephrotoxicity, particularly during high-dose treatment. This toxicity is linked to its metabolite, 7-hydroxymethotrexate, although the precise molecular mechanisms remain unclear.

Objectives: In this study, we aimed to employ molecular docking methods to compare the binding of methotrexate and 7-hydroxymethotrexate to key renal proteins—OAT1, OAT3, and DHFR.

Material and methods: In this study, we used structures of methotrexate and 7-hydroxymethotrexate to key renal proteins, OAT1, OAT3, and DHFR from AlphaFold, RCSB PDB, and PubChem. AutoDock Vina and PyMOL were used for docking and interaction analysis.

Results: Results showed that 7-hydroxymethotrexate binds more strongly to all targets, especially OAT3 (docking score: -9.7 vs. -8.6 kcal/mol), and forms unique hydrogen bonds with residues Tyr356, Ser442, and Arg469 in OAT3. While both ligands had good affinity for DHFR, differences were more pronounced for the renal transporters.

Conclusions: These findings suggest that enhanced binding of 7-hydroxymethotrexate to OAT3 may contribute to its nephrotoxic risk, supporting the importance of transporter-mediated drug interactions in methotrexate therapy.

1. Introduction

Methotrexate is a widely used antimetabolite chemotherapeutic and immunosuppressive agent, prescribed for a variety of malignancies as well as autoimmune conditions such as rheumatoid arthritis and psoriasis[1]. As a folate analogue, methotrexate exerts its cytotoxic effects by competitively inhibiting the enzyme dihydrofolate reductase, thereby disrupting DNA synthesis, repair, and cellular replication[2]. This mechanism underpins its efficacy in rapidly proliferating cells, making methotrexate a cornerstone of treatment protocols for acute lymphoblastic leukaemia, osteosarcoma, breast cancer, and several other solid tumours, as well as for the management of inflammatory diseases[3].

Despite its broad clinical utility and well-established therapeutic benefits, methotrexate administration particularly at high or repeated doses is frequently associated with a spectrum of adverse effects[4]. Among these, nephrotoxicity is particularly significant and clinically challenging[5,6]. Kidney injury not only threatens patient safety by impairing renal function but also poses a unique risk in the context of methotrexate pharmacokinetics. Since methotrexate and its metabolites are primarily eliminated via renal excretion, any compromise in kidney function can result in delayed drug clearance, increased plasma and tissue levels of methotrexate, and a heightened risk of systemic toxicity[7]. This can precipitate a cascade of secondary adverse effects, including myelosuppression, mucositis, hepatotoxicity, and neurotoxicity, which may be life-threatening if not promptly managed.

The risk of nephrotoxicity is amplified in high-dose methotrexate regimens, which are often necessary for optimal antitumor efficacy but require intensive monitoring and supportive care to prevent or mitigate

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renal complications[8]. Thus, understanding the mechanisms underlying methotrexate-induced kidney injury, as well as the factors that predispose certain patients to toxicity, remains a critical area of research to improve therapeutic outcomes and patient safety.

A primary mechanism underlying methotrexate-induced nephrotoxicity involves the drug's metabolism and elimination pathways. Following administration, methotrexate is subjected to hepatic metabolism, where it undergoes enzymatic oxidation primarily via aldehyde oxidase to produce 7-hydroxymethotrexate, its principal metabolite[8]. Unlike the parent compound, 7-hydroxymethotrexate exhibits markedly lower aqueous solubility, particularly in the slightly acidic environment of urine. This physicochemical property increases the risk of crystal formation and precipitation within the renal tubular system, a process that can obstruct tubular flow and initiate local inflammatory responses. The resultant direct tubular injury often manifests as acute kidney dysfunction, which can complicate ongoing therapy and limit the safe administration of subsequent methotrexate doses[9].

Beyond precipitation, the efficient elimination of both methotrexate and its hydroxylated metabolite is critically dependent on active secretion by renal organic anion transporters. Among these, organic anion transporter 1 (OAT1, SLC22A6) and organic anion transporter 3 (OAT3, SLC22A8) play dominant roles in mediating the uptake and clearance of methotrexate and its metabolites from the bloodstream into the renal tubular cells[10]. Any impairment in the function of these transporters whether due to genetic polymorphisms, drug interactions, or competitive inhibition by accumulated metabolites can substantially decrease the renal clearance of methotrexate and 7-hydroxymethotrexate. Such impaired excretion not only increases systemic exposure and toxicity risk but also promotes further accumulation of the less soluble metabolite in the kidney, establishing a vicious cycle that amplifies nephrotoxic potential.

The interplay between hepatic metabolism, renal transport, and urinary solubility thus forms a complex but crucial foundation for understanding methotrexate-induced nephrotoxicity. Delineating the molecular determinants of these processes, particularly at the level of transporter-ligand interactions, remains essential for improving risk prediction and developing effective preventive strategies in patients undergoing high-dose methotrexate therapy.

While the nephrotoxic effects of methotrexate and its metabolites are well documented in clinical literature, the molecular basis for their differential interactions with renal transport proteins remains incompletely understood[11]. Numerous clinical reports and pharmacovigilance data have highlighted the association between high-dose methotrexate regimens and acute kidney injury, often implicating the accumulation of 7-hydroxymethotrexate as a key risk factor. However, despite the central role of renal organic anion transporters in the excretion of both methotrexate and its metabolites, the precise mechanisms by which these transporters interact with and distinguish between structurally related compounds have not been fully elucidated. In particular, it remains unclear whether differences in binding affinity, molecular orientation, or specific residue interactions within OAT1, OAT3, and other renal proteins might account for the greater nephrotoxic potential of 7-hydroxymethotrexate compared to the parent drug.

Traditional experimental approaches, such as transporter knockdown models or in vitro uptake assays, offer valuable information but are often limited by technical complexity, ethical considerations, and the inability to resolve atomic-level interactions. Recent advancements in computational molecular docking and structural bioinformatics now enable researchers to model and visualize the detailed interactions between small molecules and protein targets, thereby providing mechanistic insights that are otherwise challenging to obtain. These in silico methods facilitate the comparison of binding affinities, prediction of key contact residues, and identification of unique interaction patterns, all of which contribute to a more comprehensive understanding of drug–transporter dynamics[12,13]. Leveraging such computational tools holds promise for elucidating the underpinnings of methotrexate-related nephrotoxicity and informing the development of targeted strategies for toxicity mitigation.

In this study, we employed molecular docking techniques to systematically compare the binding affinities and interaction profiles of methotrexate and its primary metabolite, 7-hydroxymethotrexate, with three proteins central to its pharmacology and toxicity: organic

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anion transporter 1 (OAT1), organic anion transporter 3 (OAT3), and dihydrofolate reductase (DHFR) the canonical intracellular target responsible for methotrexate's antimetabolite activity. Through in silico modeling, we sought to elucidate both the structural and energetic distinctions in how the parent drug and metabolite interact with these critical renal transporters and enzyme. By revealing differences in binding affinity, key residue interactions, and docking orientations, this work aims to advance the mechanistic understanding of why 7-hydroxymethotrexate possesses heightened nephrotoxic potential. Ultimately, these insights may contribute to the development of predictive tools for toxicity risk and guide more effective clinical strategies for the prevention and management of methotrexate-induced kidney injury.

2. Material and methods

Protein Structure Preparation

Three target proteins were selected for docking analysis: organic anion transporter 1 (OAT1; UniProt ID: Q4U2R8), organic anion transporter 3 (OAT3; UniProt ID: Q9H015), and dihydrofolate reductase (DHFR; PDB ID: 1U72). The 3D structures of OAT1 and OAT3 were retrieved from the AlphaFold Protein Structure Database[14,15], while DHFR was obtained from the RCSB Protein Data Bank[16]. All structures were inspected and pre-processed using PyMOL (version 3.1.6.1) to remove non-essential molecules, add hydrogens, and convert files to PDBQT format[17].

Ligand Structure Preparation

Methotrexate (PubChem CID: 126941) and 7-hydroxymethotrexate (PubChem CID: 149896) were chosen for docking studies. 3D conformers were downloaded from PubChem and prepared in PyMOL, ensuring proper geometry and protonation before conversion to PDBQT format[18,19].

Docking Protocol

Molecular docking was performed using AutoDock Vina [20]. Each receptor–ligand pair was docked using a grid box tailored to the specific binding region of each protein. The grid box sizes were fixed at 40 × 40 × 40 Å for all docking runs to maintain comparability, while the grid centers were as shown in *Table 1*.

Table 1. Docking grid box centers used for each protein–ligand pair.

Protein	Ligand	center_x	center_y	center_z
OAT1	Methotrexate	0.449	-1.110	4.477
OAT1	7-Hydroxymethotrexate	0.449	-1.110	4.477
OAT3	Methotrexate	-8.462	1.044	-1.626
OAT3	7-Hydroxymethotrexate	-8.462	1.044	-1.626
DHFR	Methotrexate	22.967	13.330	-0.702
DHFR	7-Hydroxymethotrexate	22.967	13.330	-0.702

Additional Vina parameters included an energy range of 4 and an exhaustiveness setting of 8. The configuration for each docking run specified the appropriate grid center and maintained all other parameters constant for consistent evaluation.

Analysis of Docking Results

The top-scoring pose for each receptor–ligand pair was selected based on binding affinity. Docking results were visualized using PyMOL, and key hydrogen bonds as well as interacting residues were identified. Bond lengths were measured, and interaction profiles were tabulated to facilitate comparison across proteins and ligands.

3. Results

Comparative Docking of Methotrexate and 7-Hydroxymethotrexate with OAT1, OAT3, and DHFR

Molecular docking studies were conducted to compare the binding affinities of methotrexate and its primary metabolite, 7-hydroxymethotrexate, with three proteins implicated in nephrotoxicity: organic anion transporter 1 (OAT1), organic anion transporter 3 (OAT3), and dihydrofolate reductase (DHFR). The calculated docking scores, reflecting predicted binding affinities, are summarized in Table 2.

Across all three protein targets, 7-hydroxymethotrexate exhibited more negative (i.e., stronger) docking scores compared to methotrexate. The greatest difference was observed for OAT3, where 7-hydroxymethotrexate displayed a binding affinity of -9.7 kcal/mol versus -8.6 kcal/mol for methotrexate. Similarly, for OAT1, the docking scores were -8.5 kcal/mol for the metabolite and -8.1 kcal/mol for the parent compound. A comparable trend was observed with DHFR, with docking scores of -9.6 kcal/mol for the metabolite and -8.9 kcal/mol for methotrexate.

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Both ligands formed several hydrogen bonds with the protein targets, but their patterns of interaction varied significantly (Table 2). Methotrexate established multiple hydrogen bonds with OAT1, notably with Ser203 (2.3 Å), Tyr141 (2.0 Å), Tyr354 (3.0 Å), and Arg466 (2.1 Å). In contrast, 7-hydroxymethotrexate formed a single hydrogen bond with Ala435 (3.5 Å) in OAT1.

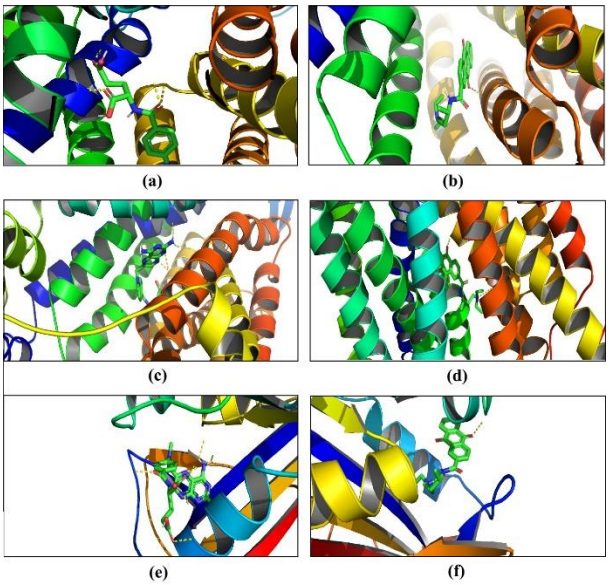

Figure 1. Docked complex visualizations of methotrexate and 7-hydroxymethotrexate with OAT1, OAT3, and DHFR

Panels (a)–(f) show the predicted binding poses obtained by molecular docking: (a) Methotrexate with OAT1; (b) 7-Hydroxymethotrexate with OAT1; (c) Methotrexate with OAT3; (d) 7-Hydroxymethotrexate with OAT3; (e) Methotrexate with DHFR; (f) 7-Hydroxymethotrexate with DHFR.

In *Figure 1*, proteins are rendered as coloured ribbons and ligands as sticks. Key interacting amino acid residues and hydrogen bonds are highlighted where applicable. The arrangement illustrates both similarities and distinct orientations in ligand binding across the three protein targets.

In OAT3, methotrexate formed hydrogen bonds with Ser465 (2.1, 2.8 Å), Thr466 (2.3 Å), and Ser157 (2.3 Å). 7-hydroxymethotrexate, however, displayed interactions with Tyr356 (2.1 Å), Ser442 (3.4 Å), and Arg469 (2.0 Å), suggesting a different binding orientation or mode.

For DHFR, methotrexate exhibited extensive hydrogen bonding, interacting with Ile7 (2.3 Å), Val115 (2.4 Å), Tyr121 (2.8 Å), Gln35 (2.1, 2.4 Å), Arg70 (2.5, 2.0 Å), and Asn64 (2.5 Å). The metabolite, however, formed a single hydrogen bond with Ser5 (3.2 Å).

Visualization of docking poses revealed that both methotrexate and 7-hydroxymethotrexate occupy similar binding pockets within each protein but adopt subtly different orientations, particularly within the OAT transporters. The additional or unique interactions of the metabolite, especially with OAT3 (Tyr356, Ser442, Arg469), may account for its higher binding affinity and altered transport properties.

Table 2. Docking Results for Methotrexate and 7-Hydroxymethotrexate with OAT1, OAT3, and DHFR

Protein	Ligand	Docking Score (kcal/mol)	Key Interactions (H-bond)	Bond Length (Å)
OAT1	Methotrexate	-8.1	Ser203, Tyr141, Tyr354, Arg466	2.3, 2.0, 3.0, 2.1
OAT1	7-Hydroxymethotrexate	-8.5	Ala435	3.5
OAT3	Methotrexate	-8.6	Ser465, Thr466, Ser157	2.1, 2.8, 2.3, 2.3
OAT3	7-Hydroxymethotrexate	-9.7	Tyr356, Ser442, Arg469	2.1, 3.4, 2.0
DHFR	Methotrexate	-8.9	Ile7, Val115, Tyr121, Gln35, Arg70, Asn64	2.3, 2.4, 2.8, 2.1, 2.4, 2.5, 2.0, 2.5
DHFR	7-Hydroxymethotrexate	-9.6	Ser5	3.2

The observed stronger binding affinities of 7-hydroxymethotrexate, particularly to OAT3, align with clinical evidence that this metabolite is more nephrotoxic. The metabolite's unique hydrogen bonds with key OAT3 residues could favour increased renal uptake or prolonged retention, leading to tubular accumulation and kidney injury. For DHFR, both ligands exhibited strong binding, but the transporters' interactions are likely more relevant for nephrotoxicity.

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4. Discussion

Methotrexate-induced nephrotoxicity remains a critical barrier to optimal dosing and safe administration in oncology and rheumatology practice, particularly during high-dose regimens that require rapid renal clearance to prevent systemic toxicity[8,21]. While both methotrexate and its major metabolite, 7-hydroxymethotrexate, are eliminated via the kidneys, clinical and preclinical data consistently implicate the metabolite as a primary driver of kidney injury due to its poor solubility and tendency to precipitate within renal tubules[22].

Our molecular docking analysis supports and extends these clinical observations by demonstrating that 7-hydroxymethotrexate exhibits consistently stronger binding affinities to both OAT1 and OAT3 compared to methotrexate. The most notable difference was observed with OAT3, where the metabolite achieved a docking score of -9.7 kcal/mol versus -8.6 kcal/mol for the parent drug. This stronger predicted binding is further substantiated by the formation of unique hydrogen bonds with residues such as Tyr356, Ser442, and Arg469 interactions absent in the methotrexate-OAT3 complex. These findings are in agreement with recent work demonstrating that OAT3, in particular, plays a dominant role in renal uptake and secretion of methotrexate and its metabolites, and that genetic or pharmacologic disruption of OAT3 function exacerbates toxicity[10,23].

Similarly, although both compounds displayed strong binding to DHFR, consistent with the canonical intracellular mechanism of action of methotrexate, the relatively modest difference in docking scores for DHFR (-9.6 vs. -8.9 kcal/mol) suggests that transporter-mediated accumulation, rather than altered target inhibition, may be more relevant to the pathogenesis of nephrotoxicity. This is further underscored by previous pharmacokinetic studies highlighting that accumulation of 7-hydroxymethotrexate in the kidney can delay methotrexate excretion, increase local toxicity, and complicate rescue therapy with leucovorin[8,21].

The present findings also align with prior in vitro studies, which showed that methotrexate and its metabolites are substrates for OAT1 and OAT3, and that impaired transporter function due to genetic polymorphisms or drug-drug interactions can significantly increase toxicity risk[24,25]. Our in-silico approach complements these studies by providing atomic-level detail on the specific residues and binding orientations that may underpin these pharmacokinetic differences.

Importantly, the unique binding pattern of 7-hydroxymethotrexate to OAT3 could mechanistically explain why this metabolite is more likely to accumulate and precipitate in renal tissues, thereby contributing to tubular obstruction and injury, as reported in clinical case series[21,26]. These results reinforce the need for close monitoring of both methotrexate and metabolite levels in patients receiving high-dose therapy, as well as consideration of renal transporter status and potential interactions with other OAT substrates or inhibitors.

5. Conclusion

This comparative molecular docking study elucidates key differences in the binding interactions of methotrexate and its major metabolite, 7-hydroxymethotrexate, with critical renal transporters (OAT1 and OAT3) and the intracellular target dihydrofolate reductase (DHFR). Our findings demonstrate that 7-hydroxymethotrexate exhibits stronger binding affinities and distinct hydrogen bonding patterns with OAT1 and OAT3 compared to the parent drug, particularly with OAT3. These results provide a structural explanation for the enhanced nephrotoxic potential of the metabolite, as increased transporter affinity may promote renal accumulation and facilitate tubular injury. The study underscores the importance of considering transporter-mediated interactions in understanding drug-induced nephrotoxicity and highlights the utility of computational docking approaches in predicting adverse drug effects. These insights may guide future strategies for individualized risk assessment, clinical monitoring, and the design of safer therapeutic agents in methotrexate-based regimens.

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