Molecular Targets

New Targets for an Old Drug

II. Hypoxanthine-Guanine Amidophosphoribosyltransferase As a New Pharmacodynamic Target of Methotrexate

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Abstract

Methotrexate has been a clinical agent used in cancer, immunosuppression, rheumatoid arthritis, and other highly proliferative diseases for many years, yet its underlying molecular mechanism of action in these therapeutic areas is still unclear. We have previously reported using a chemical proteomics technique on several other potential pharmacodynamic targets of methotrexate. Here, using a frontal affinity chromatography with mass spectrometry detection, we confirm one of these targets, hypoxanthine-guanine amidophosphoribosyltransferase, as a true binder of methotrexate with a K_d of 4.2 μM. These results complement and confirm our recent study, but more importantly, shed light into the mechanism of action of methotrexate in oncology and other highly proliferative diseases and may help explain some unaccounted for effects of this drug. For example, despite the fact that DNA salvage pathway enzymes are highly active, methotrexate can be effective if it only targets enzymes of the *de novo* pathway.

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Introduction

One of the most important concerns in drug discovery and development today is the attrition of compounds in preclinical or clinical stages. Many compounds are withdrawn at these stages because of their failure to show efficacy or tolerable toxicities *(1)*. Several technologies have started to emerge that specifically aim to address the underlying reasons for compound attrition. Among these, the development of computational tools to predict the absorption, distribution, metabolism, and excretion/toxicology (ADME)/tox behavior of compounds *(2)* and the development of chemical genomics and proteomics techniques for understanding a drug's mechanisms of action and toxicity are promising *(3)*. In particular, chemical proteomics, the capturing of a select proteome with a chemical agent or drug, is poised to have the most impact in that it directly evaluates drug–protein interactions, thus potentially revealing a drug's true mechanism of action. Several chemical proteomics applications have emerged in the recent past *(4,5)*. In particular, affinity-based methods that measure the affinity of a drug to specific proteins in a cell lysate or tissue extract under approximate physiological conditions have already yielded key mechanistic answers for several drugs *(6)*.

Methotrexate is one such drug that, despite its use for many years as a clinical agent in cancer *(7)*, immunosuppression *(8)*, rheumatoid arthritis *(9)*, and other highly proliferative diseases, still has questionable molecular mechanisms of action in several of these therapeutic areas, specially immunosuppression and rheumatoid arthritis *(10)*. In cancer, the mechanism of action of methotrexate has been understood as occurring as a result of cytotoxicity originating from the accumulation of the corresponding polyglutamated methotrexate metabolites in cells *(11)*.

Methotrexate is taken into cells by reduced folate carrier protein, where it is polyglutamated by folylpolyglutamate synthetase (FPGS). On polyglutamation, methotrexate binds to dihydrofolate reductase (DHFR), its known primary pharmacodynamic target, interrupting the conversion of dihydrofolate to the activated N5,N10-methylene-tetrahydrofolate. N5,N10-methylene-tetrahydrofolate is the main methylene donor in *de novo* purine biosynthesis; it provides the methyl group in the conversion of deoxyuridine 5'-monophosphate to deoxythymidine-5'-monophosphate for DNA synthesis and for many transmethylation processes. The polyglutamated metabolite is subject to back glutamyl hydrolysis by γ-glutamyl hydroxamate (γ-GH) and efflux from cells. Several other clinical oncology targets are known that are inhibited by methotrexate or other antifolate compounds, mainly thymidylate synthase (TS) and glycinamide ribonucleotide transformylase (GART) *(12)* and amino-imidazolecarboxamide-ribonucleotide transformylase (AICARFT) *(13)*.

The main problem with methotrexate and other classical antimetabolites is that accumulation of polyglutamated metabolites in cells causes drug resistance. Several mechanisms of resistance *(14)*, such as defective transport through cell membranes, amplification of DHFR, reduced expression of FPGS, and upregulation of γ-GH, have been identified as the underlying basis for the development of resistance to antifolates. Because of the increased resistance with current antifolate drugs, there is a need for new antifolate targets. The development of clinical diagnostic markers for antifolate drug resistant tumors would also be beneficial in deciding which therapies to choose for those tumors. Of equal importance to clinical applications is the understanding of the molecular mechanism of action and toxicity of existing and emerging antifolate therapeutics.

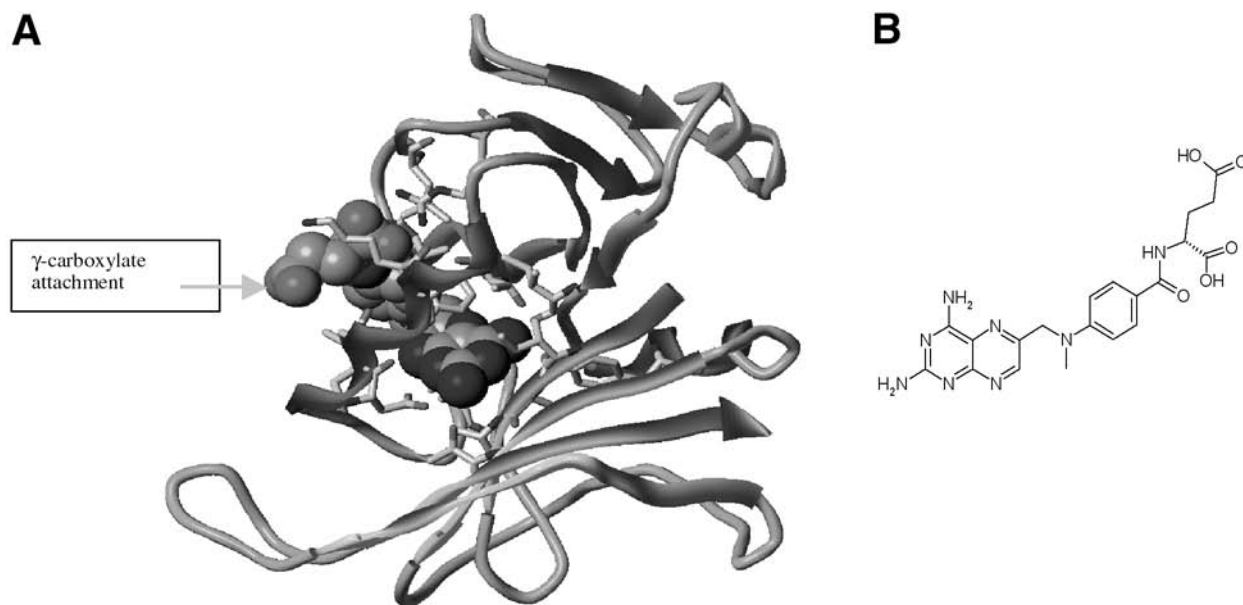We have recently reported on methotrexate's potential pharmacodynamic target profile *(15)*.

Fig. 1. **(A)** Crystal structure of methotrexate complexed within the active site of dihydrofolate reductase showing the γ-carboxylate protruding out of the cavity. **(B)** Methotrexate.

Using methotrexate, tethered to an agarose solid support in a manner consistent with binding DHFR (Fig. 1), the three known antifolate targets in the clinic, DHFR, GART, and TS, and 19 other proteins were recovered in a single affinity-capture experiment when the probe was exposed to a cell lysate. Conceptually, proteins can associate with the immobilized ligand either through a direct binding interaction or by an association with a direct binding protein. Using the protein-ligand docking algorithm Gold *(16)* in an inverse docking procedure *(17)*, the proteins were categorized as direct targets or indirect binders of methotrexate. In this inverse docking procedure, the methotrexate ligand was docked against the crystal structure of all but one of the recovered proteins, and the proposed docking poses were scored by a consensus scoring technique. Depending on the proposed binding mode and binding energy score, we categorized them as direct targets of methotrexate or indirect interactors that most likely were recovered by a secondary interaction with a direct

target. Specifically, if the proposed binding mode was similar to that observed for the methotrexate-DHFR complex and the γ-carboxylate protruded out of the cavity, consistent with binding to the affinity probe, and the binding energy score was low, the protein was considered a direct target. For several of the proteins we recovered and deemed direct binders, there was also evidence in the literature for direct inhibition by methotrexate or folate derivatives.

Here we present conclusive evidence for direct binding by one of these targets, hypoxanthine-guanine amidophoshoribosyltransferase (HGPRT), and discuss the implications of these findings. Using a frontal affinity chromatography technique coupled to mass spectroscopy detection (FAC-MS) we immobilized HGPRT to a solid support and measured its binding to methotrexate. The principles on which FAC-MS are based have been previously published elsewhere *(18)*, but briefly, this is an assay based on the continuous infusion of small molecules over a protein target that has been immobilized onto a solid

support column followed by MS detection of the elutents from the column. As ligands flow through the column, they bind to the target with differing affinities. As a result, individual ligands are retained in the column, causing an increase in their breakthrough volume, that is, the effluent volume passing through the column that allows the output ligand concentration to equal the input ligand concentration. The breakthrough volume, characterized as a sigmoidal front, can readily be detected by MS and corresponds directly to the time that the front (breakthrough time) is observed to pass through the column. As such, FAC-MS offers a very convenient way of measuring the relative binding strengths of ligands for the immobilized target. We also present here FAC-MS as a complementary method to our affinity-based chemical proteomics technique. This technique, amenable to any protein that can be immobilized onto a column format, represents a tool for confirming drug–protein interactions and measuring the strength of such interactions.

Materials and Methods

Materials

αMan(1→3)[αMan(1→6)]βManO-octyl (M3) was obtained from Calbiochem (San Diego, CA). CBX1000C controlled-pore glass beads (200/400 μm, 972-Å pore size) were purchased from Millipore (Lincoln Park, NJ). HGPRT from *Saccharomyces cerevisiae,* methotrexate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), *N*-hydroxysulfosuccinimide (NHS), 2-[*N*-Morpholino]ethanesulfonic acid (MES), and all other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

FAC-MS

CBX1000C (COOH-modified) beads (5 mg) were activated by reaction with EDAC/NHS in 0.1 M MES buffer containing 0.5 M NaCl at pH 6.4. After 45 min of mixing at room temperature, the beads were centrifuged and the supernatant was removed. Beads were resuspended in 250 μL of MES buffer and 100 mg of protein (in 1× PBS) was added. The mixture was incubated for 2 h at room temperature and overnight at 4°C with 360° vertical rotation followed by 1× PBS. After loading immobilized HGPRT, the FAC-MS capillary columns (250 μm id × 2.5 cm) were washed with 50 μL (at 200 μL/h) of 1× PBS buffer followed by 50 μL of the running buffer (20 mM NH$_4$OAc containing 1% dimethyl sulfoxide [DMSO]). The analyte solution contained methotrexate (1 μM) as the indicator and M3 (1 μM) as the void marker in 20 mM NH$_4$OAc containing 1% DMSO. The makeup buffer was 90% methanol containing 0.1% acetic acid in water. The flow rates used were 80 μL/h for the makeup buffer and 100 μL/h for the FAC-MS columns. The column was connected to an AB/Sciex API 3000 triple-quadrupole mass spectrometer (Concord, ON, Canada) and syringe pumps (Harvard Biosciences, Holliston, MA) and was allowed to equilibrate with the running buffer until the methotrexate (M+H) signal was stable, then data were acquired. After 1 min, the system was switched to the analyte solution and data collection continued until the methotrexate signal had maximized for at least 10 min. The column was washed with running buffer until the methotrexate signal had reduced to its background level to regenerate the column. The data were analyzed using a customized Excel macro to determine the breakthrough times of methotrexate and M3.

Results and Discussion

Hypoxanthine-Guanine Amidophosphoribosyltransferase

HGPRT is the most important enzyme of purine salvage pathway. It catalyzes the salvage conversion of hypoxanthine and guanine to
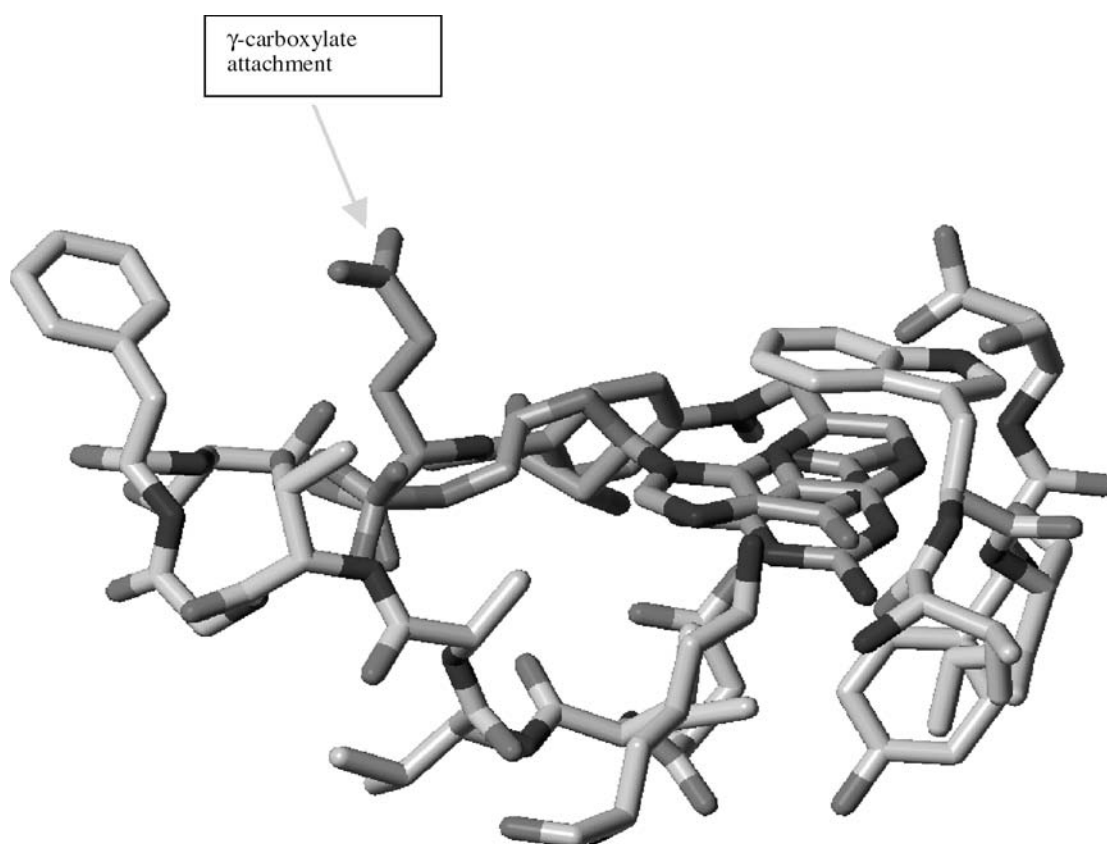
Fig. 2. Proposed binding mode for methotrexate in hypoxanthine-guanine amidophosphoribosyltransferase.

inosine 5'-monophosphate (IMP) and guanine 5'-monophosphate (GMP) respectively, by facilitating the addition of the bases to the activated phosphoribosylpyrohosphate (PRPP) molecule *(19)*. HGPRT had been previously identified by our earlier affinity-capture experiments from a cell lysate as a potential binder to methotrexate. Our inverse docking experiments were consistent with direct binding, as we found several low energy binding modes for methotrexate showing a good fit in the HGPRT binding pocket and with the glutamate group protruding out of the cavity, consistent with binding the probe directly. In these binding modes, methotrexate shows a good overlap (Fig. 2) with the experimental position occupied by the hypoxanthine monophosphate cofactor from the crystal structure complex of HGPRT with hypoxanthine monophosphate (pdb code: 1D6N [20]). There is also evidence from the literature indicating that HGPRT should be a direct target of methotrexate. For example, it is known that other enzymes that bind IMP can also be subject to inhibition by folates and methotrexate *(21)*. Furthermore, it is also known from extensive medicinal chemistry work on antimetabolite drug research that the pterin group of methotrexate can be replaced with xanthine and xanthine-like moieties and *vice versa*. Examples of this are pemetrexed (ALIMTA, LY-231514), the classical antimetabolite TS inhibitor drug from Lilly *(22)*, and tomudex (ZD9331), the nonclassical TS inhibitor from AstraZeneca *(23)*.

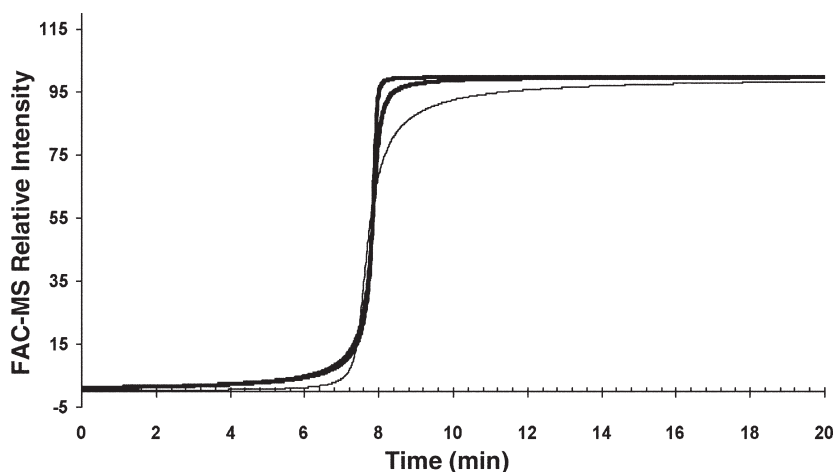The activity of salvage enzymes like HGPRT is higher than the activity of enzymes

Fig. 3. Frontal affinity chromatography-mass spectroscopy (FAC-MS) chromatogram of nonspecific binding of methotrexate to CBX beads and CBX beds with immobilized bovine serum albumin (thick lines). The thin line represents the void marker (M3).

involved in the *de novo* purine pathways, such as GART, AICARFT, and DHFR, the known targets of methotrexate *(24)*. It has been puzzling that agents such as methotrexate and other antimetabolites, believed to act primarily on *de novo* enzymes, are effective despite the presence of highly active salvage enzymes. This has recently been partly accounted for by new observations showing that methotrexate can reduce the activity of HGPRT *(25)*. Other observations suggest in vivo inhibition of HGPRT by methotrexate. For example, deficiency in HGPRT is known to result in higher levels of PRPP and an acceleration of purine biosynthesis by the *de novo* pathway *(26)*. Treatment with methotrexate also produces an increase in levels of PRPP *(27)* and this effect is reversible on treatment with hypoxanthine.

In order to confirm HGPRT as a direct pharmacodynamic target of methotrexate, we immobilized it on a solid support by covalent attachment. To evaluate potential binding of methotrexate, we first examined its MS signal. A molecular ion $(M+H)^+$ 455.5 corresponding to the molecular weight of 454.5 showed strong intensity, good stability, and dynamic range. Nonspecific interactions of methotrexate were tested with blank columns packed with nonmodified controlled-pore glass (CBX) beads and with columns packed with bovine serum albumin (BSA) immobilized beads (Fig. 3). The breakthrough times of methotrexate for both columns was determined to be minimal (approx 0.2 min), indicating that methotrexate has very little nonspecific binding to both CBX beads and to neutral protein. These features make methotrexate an excellent indicator for FAC-MS experiments. After immobilization of HGPRT onto the beads, confirmation of FAC-MS activity was obtained, as shown in Fig. 4, by seeing significant binding (delayed breakthrough time of 56 min compared with the void marker, M3 at 9 min) of methotrexate at a concentration of 1 μ*M*. These data, along with very low nonspecific binding, indicate that methotrexate is truly binding to HGPRT. Using the direct method (staircase) *(28)*, we determined (data not shown) that the K_d of methotrexate for HGPRT is 4.2 μ*M*. We also believe that this binding would be preserved with HGPRT derived from mammalian sources.
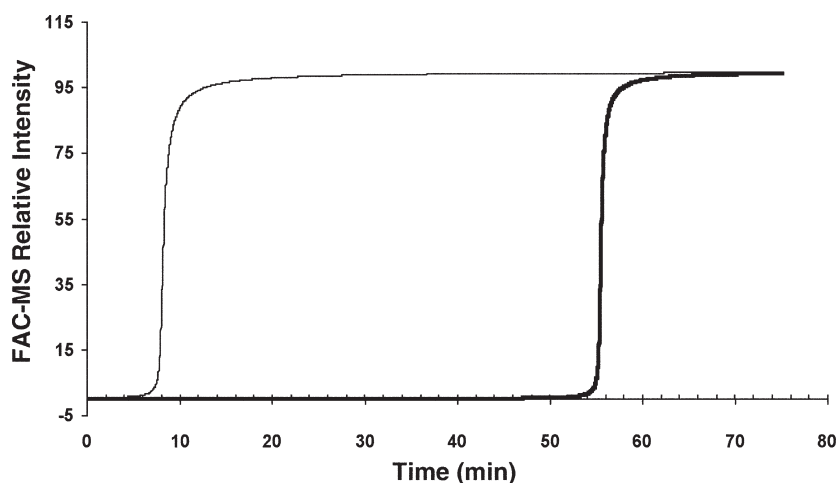
Fig. 4. Frontal affinity chromatography-mass spectroscopy (FAC-MS) chromatogram of binding of methotrexate to CBX beads with immobilized hypoxanthine-guanine amidophosphoribosyltransferase. The thick line represents the void marker and the thin line represents methotrexate.

Conclusions

We have recently shown by a chemical proteomics experiment that when methotrexate is immobilized on a solid support in a manner compatible with binding its known target DHFR, and exposed to a cell lysate, many other proteins associated with *de novo* and salvage purine biosynthetic pathways are captured. Three of these, DHFR, GART, and TS, are already known as pharmacodynamic targets of this drug or other antimetabolites, respectively. Using an inverse protein-ligand docking technique and evidence from the literature, we had proposed that at least 9 of the 21 identified proteins are potential direct targets of methotrexate. In the current experiment, we make use of FAC-MS to confirm our earlier results and provide conclusive evidence for direct binding of methotrexate by one of these targets, HGPRT. This target binds methotrexate with a K_d of 4.2 μM. Our findings and evidence from the literature indicate that direct inhibition of HGPRT by methotrexate is at least in part responsible for methotrexate's efficacy as an anticancer agent and other highly proliferative diseases.

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