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Interspecies differences in the metabolism of methotrexate: An insight into the active site differences between human and rabbit aldehyde oxidase

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ABSTRACT

Several drug compounds have failed in clinical trials due to extensive biotransformation by aldehyde oxidase (AOX) (EC 1.2.3.1). One of the main reasons is the difficulty in scaling clearance for drugs metabolised by AOX, from preclinical species to human. Using methotrexate as a probe substrate, we evaluated AOX metabolism in liver cytosol from human and commonly used laboratory species namely guinea pig, monkey, rat and rabbit. We found that the metabolism of methotrexate in rabbit liver cytosol was several orders of magnitude higher than any of the other species tested. The results of protein quantitation revealed that the amount of AOX1 in human liver was similar to rabbit liver. To understand if the observed differences in activity were due to structural differences, we modelled rabbit AOX1 using the previously generated human AOX1 homology model. Molecular docking of methotrexate into the active site of the enzyme led to the identification of important residues that could potentially be involved in substrate binding and account for the observed differences. In order to study the impact of these residue changes on enzyme activity, we used site directed mutagenesis to construct mutant *AOX1* cDNAs by substituting nucleotides of human *AOX1* with relevant ones of rabbit *AOX1*. AOX1 mutant proteins were expressed in *Escherichia coli*. Differences in the kinetic properties of these mutants have been presented in this study.

1. Introduction

Aldehyde oxidase (AOX) (EC 1.2.3.1) is a molybdoflavoenzyme that is active as a homodimer comprised of two identical subunits of 150 kDa each. Two iron sulphur [2Fe2S] clusters, FAD and a molybdenum cofactor domain are essential constituents of AOX catalytic activity [1]. As the name suggests, AOX is capable of oxidising aldehydes to their corresponding carboxylic acids. In addition, it also metabolises iminium ions and aromatic azaheterocycles. The mechanism of action of AOX involves the nucleophilic addition of molybdenum hydroxide to an electron deficient carbon, followed by a hydride transfer from the substrate to reduce the molybdenum cofactor [2,3]. In this process the formal charge on molybdenum is reduced from +VI to +IV. Electron transfer to molecular oxygen is mediated via the two [2Fe-2S] clusters and a flavin adenine dinucleotide. The molybdenum coordinated product that is formed is released by displacement with water.

Since the aromatic azaheterocycles comprise a substructure of many drug compounds, biotransformation of these compounds by AOX is the most relevant to drug metabolism. Currently very few drugs such as the sedative zaleplon [4], antipsychotic ziprasidone [5] and anti cancer agents such as zebularine [6] and methotrexate [7] are primarily cleared by AOX. However, this number is expected to increase as efforts to reduce cytochrome P450 liability in combination with the industry's move into new drug space with kinase inhibitors is resulting in an increase in the development of compounds containing the typical azaheterocyclic structural moiety [8]. To date, conventional animal models employed to predict clearance in humans have not been successful in the case of AOX, although a computational model has been successfully utilised [9]. Use of preclinical animal models to predict human clearance, which has been successfully applied in the case of cytochrome P450 mediated metabolism, has failed for AOX because of substrate dependent species differences in AOX

Abbreviations: AOX1, aldehyde oxidase 1; mAOX3, mouse aldehyde oxidase 3.

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metabolism [10–12]. This means that a single preclinical species cannot be used indiscriminately for predicting clearance of all AOX substrates in human.

In humans, the antineoplastic agent methotrexate, 4-amino-N^{10}-methylpteroglutamic acid, has been widely used in the treatment of several malignancies and autoimmune disorders and is primarily cleared through renal excretion [13]. It is known that cytochrome P450s do not contribute to the metabolism of this drug [14]. AOX is the primary drug metabolising enzyme responsible for the formation of the highly insoluble metabolite, 7-hydroxymethotrexate, which is believed to be involved in toxicity by causing renal dysfunction due to precipitation in the renal tubule [7,15]. In addition, it is also thought to interfere with the pharmacokinetics of methotrexate in vitro, thereby affecting its cellular entry and efflux [16]. At conventional dose levels of methotrexate in primates, 7-hydroxymethotrexate was not detected in vivo. However, with high dose (>50 mg methotrexate/kg) therapy regimes employed for the treatment of certain cancers as high as 33% of the parent compound can be excreted as 7-hydroxymethotrexate [15]. This suggested that the conversion of methotrexate to 7-hydroxymethotrexate is a dose dependent phenomenon with the enzyme involved having low affinity for methotrexate. Indeed, human AOX1 has a very low affinity for methotrexate [7]. Rabbit AOX on the other hand has been reported to have extremely high AOX activity with methotrexate [17,18]. In order to understand the structural determinants responsible for high methotrexate activity, a rabbit homology model was generated and methotrexate was docked in to its active site. Using site directed mutagenesis, we exchanged amino acid residues found in the active site of human AOX1 with the ones found in rabbit AOX1.

2. Materials and methods

2.1. Chemicals used and enzyme source

Methotrexate and the metabolite 7-hydroxymethotrexate were purchased from Sigma Aldrich (St. Louis, MO) and Toronto Research Chemicals (Toronto, Ontario, Canada) respectively. Animal liver cytosol was purchased from Xenotech LLC (Lenexa, KS) whereas human liver cytosol was bought from BD Biosciences (Franklin Lakes, NJ). Sequence grade trypsin was purchased from Promega (Madison, WI). The synthetic peptide standard (H-Met-Tyr-Lys-Glu-Ile*-Asp-Gln-Thr-Pro-Tyr-Lys-Gln-Glu-NH_2 with heavy isotope labelling Ile* = U-$^{13}C_6$) used for quantitation of AOX levels in monkey and rabbit was acquired from Anaspec (Fremont, CA). Table 1 lists information about the species liver cytosols used in this study.

2.2. Incubation conditions

Incubation mixtures consisted of 1–500 μM methotrexate in 25 mM potassium phosphate buffer (pH 7.4), containing 0.1 mM EDTA. Methotrexate stocks were made up in dimethyl sulfoxide (DMSO) and added to the incubation mixture such that the total concentration of DMSO in each sample was 0.5% (v/v).

Table 1
Information about liver cytosol used in this study.

Species	Livers pooled	Sex
Human	150	Mixed
New Zealand Rabbit	6	Male
Sprague Dawley Rat	433	Male
Hartley Albino Guinea Pig	50	Male
Rhesus Monkey	6	Male

Incubations were performed in a 37 °C water bath incubator. Production of 7-hydroxymethotrexate was optimised with respect to time and protein concentration to establish initial velocity conditions for kinetic measurements. The reactions were initiated by the addition of prewarmed liver cytosol at a final concentration of 10 mg of total protein/ml of reaction for human liver cytosol, 5 mg/ml for rat and guinea pig liver cytosol and 0.5 mg/ml for monkey and rabbit liver cytosol. The final incubation volume was 100 μl. Human, monkey, rat and guinea liver cytosol was incubated with methotrexate for a total of 180 min whereas rabbit liver cytosol was incubated with methotrexate for 20 min. The reaction was quenched with 20 μl of 1 M formic acid containing 20 μg/ml of the internal standard, 2-methyl-4(*3H*)-quinazolinone. Quenched samples were centrifuged at 5000 rpm for 10 min in a 5415D Eppendorf centrifuge to precipitate protein content and the collected supernatant was analysed by LC–MS/MS.

2.3. Liquid chromatography–mass spectrometry conditions

An Agilent 1100 series high-performance liquid chromatography system (Agilent technologies, Santa Clara, CA) coupled to an API4000 triple quadrupole mass spectrometry system manufactured by Applied Biosystems/MDS Sciex (Foster City, CA) was used. Samples were analysed in positive ion mode using an electrospray ionisation interface. Chromatography was performed on a Synergi Polar reverse-phase column (30 mm × 3.0 mm, 4 μM; Phenomenex, Torrance, CA). Mobile phase A consisted of 0.05% formic acid and 0.2% acetic acid in water and mobile phase B comprised of 90% acetonitrile, 9.9% water and 0.1% formic acid.

2.4. Metabolite quantification

Before sample injection, the column was equilibrated with 95% mobile phase A for 1 min. Chromatographic separation was achieved using a linear gradient over the next 2.5 min to 5% mobile phase A. Mobile phase A was then held constant at 5% for the next 0.5 min followed by a linear gradient to 95% A over the next 0.5 min. Finally, the column was re-equilibrated to initial conditions over the last 0.5 min. Total chromatographic assay time was 5 min per sample with a flow rate of 800 μl/min and the retention time per sample for the internal standard and the metabolite were 1.5 and 3 min respectively.

The optimised mass spectrometry tune parameters for 7-hydroxymethotrexate were as follows: collision gas, 4; curtain gas, 10; ion source gas 1,50; ion source gas 2,5; ion spray voltage, 5000; desolvation temperature, 350; declustering potential, 60; entrance potential, 15; collision energy, 35; cell exit potential, 15.

The analyte, 7-hydroxymethotrexate and the internal standard [2-methyl-4(3H)-quinazolinone] were detected using multiple reaction monitoring mode by monitoring the *m*/*z* transition from 471 to 191 and 161 to 120 respectively. The product obtained was quantified from a standard curve ranging from 10 nM to 10 μM of 7-hydroxymethotrexate. The LC–MS/MS, and incubation conditions for DACA and phthalazine were described by Barr [19,20].

2.5. AOX1 protein quantitation using LC–MS/MS

Quantitation of AOX protein in monkey and rabbit liver was carried out as previously described [21]. Liver cytosol (25 μl of the 10 mg/ml stock) was mixed with an equal volume of denaturing buffer containing 8 M urea and 2 mM DTT (4 M urea and 1 mM DTT final concentration). The reaction mixture was incubated at 60 °C for 60 min followed by subsequent dilution with 25 mM sodium bicarbonate buffer (pH 8.4) containing 100 nM peptide internal

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