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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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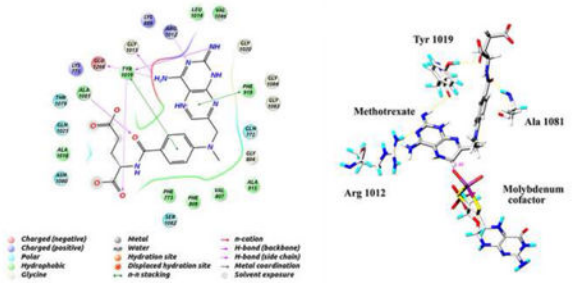
Abstract

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 *E. coli*. Differences in the kinetic properties of these mutants have been presented in this study.

Graphical Abstract



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Chemical Compounds: Methotrexate (PubChem CID: 5484402); 7-hydoxymethotrexate (PubChem CID: 126941)

Keywords

Aldehyde Oxidase; Methotrexate; Drug Metabolism; Enzyme Kinetics; Homology Modelling and Site Directed Mutagenesis

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 has 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 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).

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 minutes whereas rabbit liver cytosol was incubated with methotrexate for 20 min. The reaction was quenched with 20 μl of 1M 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 x 3.0 mm, 4 μM; Phenomenex, Torrence, 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 [19]. Liver cytosol (25 μl of the 10 mg/ml stock) was mixed with an equal volume of denaturing buffer containing 8M urea and 2 mM DTT (4M urea and 1 mM DTT final concentration). The reaction mixture was incubated at 60°C for 60 minutes followed by subsequent dilution with 25 mM sodium bicarbonate buffer (pH 8.4) containing 100 nM peptide internal standard. 10 μl of 0.5 μg/ml trypsin solution was added to the cytosol such that the ratio of trypsin to cytosol was 1:50. The mixture was incubated overnight at 37°C and reaction quenched by adding freshly prepared 50 % v/v solution of trifluroacetic acid (TFA) in water such that the final concentration was 10 % TFA v/v. Samples were subsequently vortexed and centrifuged at 1460g for 10 min prior to LC-MS/MS analysis. Digested samples were analysed by LC-MS/MS in positive ion mode. Chromatographic separation was achieved on a HALO C18 column (2.1 x 150 mm, 2.7 μm; Advanced Materials technology, Wilmington, DE)

2.6 Homology modelling and substrate docking

Human AOX1 homology model [10] previously generated from the template structure of mouse AOX3 (PDB: 3ZYV) [20], using Schrödinger's prime module (Schrödinger,

Portland, Oregon), was used to create the rabbit homology model. Sequence alignment was performed using ClustalW with primary sequences of human and rabbit AOX1 showing 84 % homology. Homology modelling to generate the protein structure of rabbit AOX1 was followed by induced fit docking workflow using methotrexate as a ligand. Finally, creating a bond to the 7-position of methotrexate from the Moco-OH formed the tetrahedral intermediate of methotrexate. This structure was minimised and the amino acids residues in close contact with the methotrexate were determined.

2.7 Site directed mutagenesis and purification of AOX1 mutants

Human AOX1 mutants were made using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The forward and reverse primers used are listed in Table 2. Double mutant cDNAs were created from cDNA with the single mutation and using mutagenic primers for the second desired mutation. This double mutant cDNA was used for the creation of the triple mutant. The resulting mutant PQE-30 Xa (QIAGEN, GmbH, Hilden, Germany) constructs were verified by sequence analysis and transformed into competent *E. coli* TP1000 cells [21]. Wild type human AOX1 and the mutants were overexpressed as an N-terminal hexa–His tagged fusion protein in TP-1000 *E. coli* cells. Cells were lysed and protein was purified using a 1 ml HiTrap Chelating HP column charged with Ni^{2+} (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Subsequently, the purified protein was dialysed into 100mM potassium phosphate buffer, pH 7.4 and stored at −80°C until further analysis [22].

3. Results

3.1 Quantitation of AOX1 protein levels in cytosol using LC-MS/MS

In order to determine AOX1 expression levels in human, rabbit and monkey liver cytosol, protein quantitation was carried out using a method developed previously in our laboratory [19]. No significant difference ($P < 0.05$) was found in the expression of AOX1 between the three species. Expression levels were in the range of 10 to 20 pmol/mg protein. To determine if these levels (Figure 1A) correlated with observed activity, they were plotted against V_{max} (Figure 1B). As shown in Figure 1B, no correlation between AOX1 expression levels and methotrexate hydroxylation activity was observed. Furthermore, it was not possible to determine the absolute amount of protein for the other species since they do not have the same sequence of the peptide fragment we used for our LC-MS/MS assay.

3.2 Interspecies differences in methotrexate hydroxylation activity in liver cytosol

Since the incubation time for the reaction was 3 hours for all samples except rabbit cytosol, it was necessary to ensure that metabolite formation observed was due to an enzyme catalyzed reaction and not auto oxidation. For this purpose, MTX was incubated for 3 hours in the presence and absence of the enzyme and with heat inactivated enzyme. Negligible amounts of metabolite were formed in the absence of enzyme, or in the presence of heat inactivated enzyme, suggesting that auto oxidation if any was a minimal contributor to the enzyme kinetics observed. This was further supported by the fact that increasing protein concentration resulted in an increase in the formation of the metabolite. Data obtained for methotrexate hydroxylation activity from liver cytosol of human, rabbit, monkey, rat and

guinea pig were fitted to the classic Michaelis-Menten equation. Since saturation of the enzyme was incomplete at concentrations close to the solubility limit, parameters, V_{max} and K_m are estimates obtained by fitting data to non linear regression analysis (Figure 2). In contrast, rabbit liver cytosol was saturated at less than 500 μM. (It was necessary to limit the methotrexate concentration used to 500 μM due to solubility concerns.) Rabbit and rhesus monkey liver cytosol had methotrexate hydroxylation activity that was substantially higher than all other species tested. The rate of methotrexate turnover (V_{max}) in rabbit liver was 1,460 times that of human liver whereas rhesus monkey metabolised methotrexate at a rate that was eight times higher than human (Table 3). The rate of methotrexate activity in guinea pig, rat and human liver was in the range of 40–70 pmol/min/mg of total protein. The rabbit liver enzyme had the highest affinity towards methotrexate as demonstrated by a K_m of 0.1 mM in comparison with 2.8 mM human liver. The Vmax/[AOX1] (k_{cat}) takes into consideration the absolute levels of AOX1 in liver cytosol that were measured by the method of Barr et al [19]. Methotrexate turnover (k_{cat}) by rabbit, rhesus monkey and human liver AOX1 was 5065, 35 and 2.4 min^{-1} respectively. Clearance due to AOX1 was calculated using specific activity values and the K_m values, and it was found that rabbit liver AOX1 was the most efficient in the clearance of methotrexate followed by rhesus monkey and human (Table 3). To explore if the difference in metabolism between rabbit and human were specific to methotrexate we also determined the k_{cat} and K_m for the substrates DACA and phthalazine as described by Barr et. al. [19, 20]. The metabolism of DACA by both rabbit and human cytosol gave similar kinetic values for k_{cat} of 182 and 133 min^{-1} and K_m 2 and 7 for rabbit and human respectively. However, rabbit metabolism of phthalazine showed a much higher kcat of 16,000 min^{-1} compared to 262 min^{-1} for humans and a slightly higher K_m value of 23 versus 8. Thus, rabbit appears to have the ability to much more rapidly oxidize specific substrates (phthalazine and methotrexate) relative to humans. The structural basis for this substrate specificity is presently being explored. However, we have chosen to focus on the differences in methotrexate metabolism between human and rabbit since they are the largest differences of the three substrates and it has clinical relevance.

3.3 Modelling of the methotrexate tetrahedral intermediate in the active site of rabbit AOX1

Since quantitative differences in AOX1 expression levels could not explain the observed interspecies differences in activity, we decided to investigate the role played by structural determinants. For this purpose, a homology model of rabbit AOX1 was modelled from the human AOX1 structure [10]. We did not use the already published mouse AOX3 structure [20] directly since the active site was too small to bind methotrexate. The primary sequence identity between human AOX1 and mouse AOX3 and human AOX1 and rabbit AOX1 was 79 % and 84 % respectively. Proteins with greater than 20 % homology are normally considered to have similar tertiary structure [23]. The tetrahedral intermediate of methotrexate after neucleophilic addition of the hydroxide from molybdenum cofactor to methotrexate was modelled in the active site of rabbit AOX1. Since this is an intermediate in the reaction sequence, the model reflects the critical interactions between the transition state of methotrexate and the protein which could possibly be involved in k_{cat} differences. Figure 4A is a two dimensional ligand interaction diagram showing residues in the substrate binding pocket of rabbit AOX1 and highlighting the interactions that stabilise the position of methotrexate in the rabbit active site. Methotrexate is buried in the region of the substrate

binding pocket containing hydrophobic residues Phe773, Phe808 Val807, Ala915, Phe919, Tyr1019. The charged residues Arg 1012 and Glu 1266 interact with nitrogen atom in the azaheterocyclic ring via a hydrogen bond whereas oxygen atoms in the side of methotrexate interact with polar residues Asn1080, Gln1021 and Thr1079.

Human AOX1 has only three residues in the active-site that are different from rabbit AOX1. Specifically (using the rabbit numbering), Arg1012 is replaced by valine, whereas Ala1081 and Tyr1019 are respectively replaced by isoleucine and alanine (Figure 3). Figure 4B shows the interaction of methotrexate with these three residues and with the molybdenum cofactor of rabbit aldehyde oxidase enzyme.

3.4 Methotrexate hydroxylation by wild type and mutant human AOX1

Given the differences between the human and rabbit enzyme, site directed mutagenesis was performed to construct mutant *AOX1* cDNAs by substituting nucleotides of human *AOX1* with relevant ones of rabbit *AOX1*. Human I1085A, A1023Y and V1016R single mutant and 11085A+A1023Y and I1085A+V1016R double mutants were made. The I1085A+A1023Y +V1016R triple mutant cDNA was generated but we were unable to express and purify sufficient amounts of protein for analysis. Expressed and purified AOX1 mutants were quantified using LC-MS/MS based protein quantitation described previously. Data obtained from methotrexate hydroxylation activity of purified recombinant mutant AOX1 were fitted using Michaelis-Menten equation to estimate the kinetics parameters, k_{cat} and K_m. None of the mutants gained the activity of the rabbit enzyme indicating that more global changes in the enzyme structure are responsible for the large difference in activity. Figure 5 shows a bar graph of the kinetic parameters for methotrexate hydroxylation by mutant and wild type AOX1. Statistical data analysis was performed using one- way ANOVA test. The I1085A single mutant had slightly higher activity compared to the wild type whereas A1023Y and V1016R showed significant decrease ($p < 0.01$) in methotrexate hydroxylation activity suggesting that Ala1023 and Val1016 were critical for human methotrexate oxidase activity. Construction of an I1085A + A1023Y double mutant did not result in a significant increase in activity compared to the I1085A single mutant. In fact, catalytic turnover of methotrexate by the double mutant was similar to the I1085A single mutant. On the other hand, the I1085A+ V1016R double mutant resulted in a significant decrease in activity compared to the wild type and I1085A. Only the I1085A single mutant and the I1085A + A1023Y double mutant had lower K_m and higher affinity whereas all other mutants showed decreased affinity for methotrexate compared to wild type AOX1.

4. Discussion

AOX is gaining increased importance as a drug metabolising enzyme because efforts to reduce cytochrome P450 metabolism have resulted in the generation of compounds with nitrogen containing aromatic ring. Such azaheterocycles have the potential to be AOX substrates [8]. However, unlike P450, AOX mediated drug metabolism is not very well understood. Conventional methods used to predict P450 metabolism in human from preclinical animal model data have failed in the case of AOX. A major reason for this is interspecies differences in AOX activity [24]. Mice, rats and dogs generally used in the prediction of P450 metabolism fail to accurately predict AOX metabolism because they

expresses a different complement of AOX isoforms in liver. For example, rodents such as mice and rat express AOX1 and AOX3 in the liver whereas dogs express none. Humans, on the other hand, express a single AOX1 isoform [25]. Since AOX activity within the same species is also dependent on the substrate used, species expressing a single AOX1 in the liver cannot be used to reliably predict AOX metabolism for every substrate [10]. For various reasons, in vitro data can only establish a rank order correlation with in vivo clearances [26]. This complicates the situation for preclinical prediction of AOX mediated human in vivo clearances. In the past, we have reported computational models for predicting AOX sites of metabolism and clearances based on the stability of tetrahedral intermediate of the substrate [9, 27]. However, these computational models suffer from their inability to predict if a compound will be an AOX substrate, and only predict rates or regioselectivity. Furthermore, since these models do not explicitly include the contribution of the enzyme active site, they cannot describe interspecies differences in clearance. In order to understand how to better predict AOX clearances in human, we need to understand the structural determinants of substrate binding, and the species specific differences in AOX binding and metabolism of potential drugs.

Methotrexate is often used as an in vitro probe substrate for the determination of aldehyde oxidase activity. Our findings suggest that methotrexate was rapidly cleared by rabbit liver followed by monkey, guinea pig, rat and human. After normalisation for protein concentration, rabbit liver AOX1 showed specific activity that was approximately three orders of magnitude higher than human liver AOX. Previous studies have reported that methotrexate hydroxylase activity in rabbit liver cytosol is approximately 2 orders of magnitude higher than human [28]. Kinetic measurements with species liver cytosol revealed that only rabbit liver AOX could be saturated before reaching the solubility limit of methotrexate, whilst saturation was not achieved with other species including human liver cytosol (Figure 2). Given this, the absolute k_{cat} values for all species except rabbit may have significant error. Even with this qualification, it appears that the rabbit enzyme is significantly faster than the human and the monkey enzyme. This difference in rates is most likely a result of the enzyme's ability to transfer electrons to oxygen or a difference in product release from the binding site. At sub-saturating concentrations the differences in k_{cat}/K_m reflect differences in affinity as well as rate. Analysis of the K_m values alone indicate that rabbit and rhesus monkey bind methotrexate with similar affinity, while human AOX has a much lower affinity for methotrexate. Either specific changes in the active site residues or global differences in the shape of the active site could explain these differences in activity.

Since no correlation was observed between expression levels of AOX1 and methotrexate hydroxylation activity, a comparison of the differences in structure between the rabbit and human enzyme might help to elucidate the differences in binding affinity and rates of reaction. Modelling of tetrahedral intermediate of methotrexate in rabbit AOX1 active site led to the identification of key interactions as shown in the two dimensional ligand interaction diagram in figure 4A. Rabbit AOX1 residues R1012, Y1019, and A1081 are replaced by valine, alanine and isoleucine respectively in human AOX1. (The human amino acid numbering for these residues is 1016, 1023, and 1085.) A study by Fukiya et al [29] compared rabbit and monkey AOX1 mutants for their activity towards chinchonidine.

Chinchonidine like methotrexate is a bulky azaheterocyclic compound and unlike monkey AOX1, rabbit enzyme has extremely high activity towards it. The authors showed that alanine 1081 (rabbit numbering that corresponds to 1085 in human and monkey) of rabbit AOX1 was essential for chinchonidine oxidation activity since mutation of this residue to the corresponding residue in monkey AOX1 (A1081V) lead to the loss of catalytic activity of rabbit AOX1 towards this substrate. In contrast, the reciprocal monkey V1085A mutant acquired high chinchonidine oxidation activity. In order to study the importance of the abovementioned residues on the activity of human AOX1, site directed mutagenesis was used to convert the human AOX1 residues to the corresponding rabbit amino acids. Mutants were expressed in *E. coli* alongside the wild type human AOX1. The K_m value for wild-type recombinant enzyme is 0.2 mM and for the enzyme from human cytosol is 2.8 mM, the latter being approximately 10 times greater than the recombinant protein. One possible explanation given the poor solubility of methotrexate is that it might bind non-specifically to cytosolic protein resulting in more substrate molecules required to achieve maximum activity and thus increasing the K_m value. The k_{cat} values for methotrexate in purified wild type human AOX1 protein are 0.8 min^{-1} whereas those for the native cytosolic enzyme are 2.4 min^{-1}. This is almost certainly a result of poor cofactor incorporation in the *E. coli* expressed enzyme [30] and is consistent with our previous results [19]. Apart from the human I1085A (human numbering) single mutant and I1085A + A1023Y double mutant, all other purified variants showed decreased specific activity in comparison to the wild type purified human AOX1. Similar to the observations made by Fukiya et al 2010 we found that human I1085A single mutant had higher specific activity and a lower K_m compared to the wild type. The mutant I1085A had a K_m that was similar to cytosolic AOX from rabbit liver, however, the increase in activity was not comparable to the difference in methotrexate activity seen between human and rabbit liver cytosol. Clearly, other factors, besides these three residues, contribute to the differences in k_{cat} values. It could be that residues remote from the active-site affect the ability of the enzyme to transfer electrons to oxygen, trapping the human enzyme in the reduced state and slowing the rate under conditions in which substrates are saturated. This type of effect would likely not be substrate dependent and rabbit should more efficiently catalyse all AO mediated reactions. In fact, Rashidi et al. [31] found that xanthine, phthalazine, phenanthridine, famciclovir, and 6-deoxypenciclovir were all metabolised at faster rates by rabbit than human partially purified AOX enzymes.

Another consideration is that human and rhesus monkey express a single AOX1 enzyme in the liver, while rodents expresses multiple AOX enzymes [25]. Analysis of the Uniprot database indicates that in addition to AOX1, rabbits have AOX3, AOX3L1 and AOX4 (Uniprot: M1ZMP8; M1ZML0 and G1TY33 respectively) at the transcript levels whereas only AOX1 (Uniprot: P80456) has been confirmed at the protein level. If rabbits are similar to rodents, both AOX1 and AOX3 may be expressed at significant levels in the liver. Thus, the differences in activity could be a result of AOX3 being the major enzyme responsible for methotrexate metabolism. This illustrates a major problem associated with structure function relationships with rats, mice, and rabbits in that multiple enzymes with much lower sequence homology may play a role in metabolism. Some light might be shed on this issue by looking at the differences in the rat versus the rabbit enzymes. One might expect rat and rabbit to have more similar kinetic constants if AOX3 is the major enzyme involved in

methotrexate metabolism and if they share a common active-site. However, rat actually appears to be closer to human when it comes to methotrexate metabolism than rabbit (Table 3).

Homology model construction of rat and rabbit AOX3 was used to show a four amino acid difference in the active-site residues. Using rat numbering the differences are H987Y, G1056A, G1057A and T1059A. It is possible that these differences in active-site structure lead to the profound differences in methotrexate metabolism. We are continuing to pursue the structure-function relationship for different species that metabolise methotrexate and we hope to have a clearer picture in the near future.

In conclusion, methotrexate is poorly metabolised by human AOX1, in contrast to rabbit. Expression levels of AOX1 in human, rhesus monkey and rabbit liver are not significantly different from each other and do not correlate with differences in activity in liver cytosol. None of the mutants created in this study could reproduce the high k_{cat} observed for rabbit cytosol. Clearly other factors contribute to the full magnitude of differences seen between the activities of the two enzymes in their native environment.

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Abbreviations

AOX1	Aldehyde oxidase 1
mAOX3	mouse aldehyde oxidase 3

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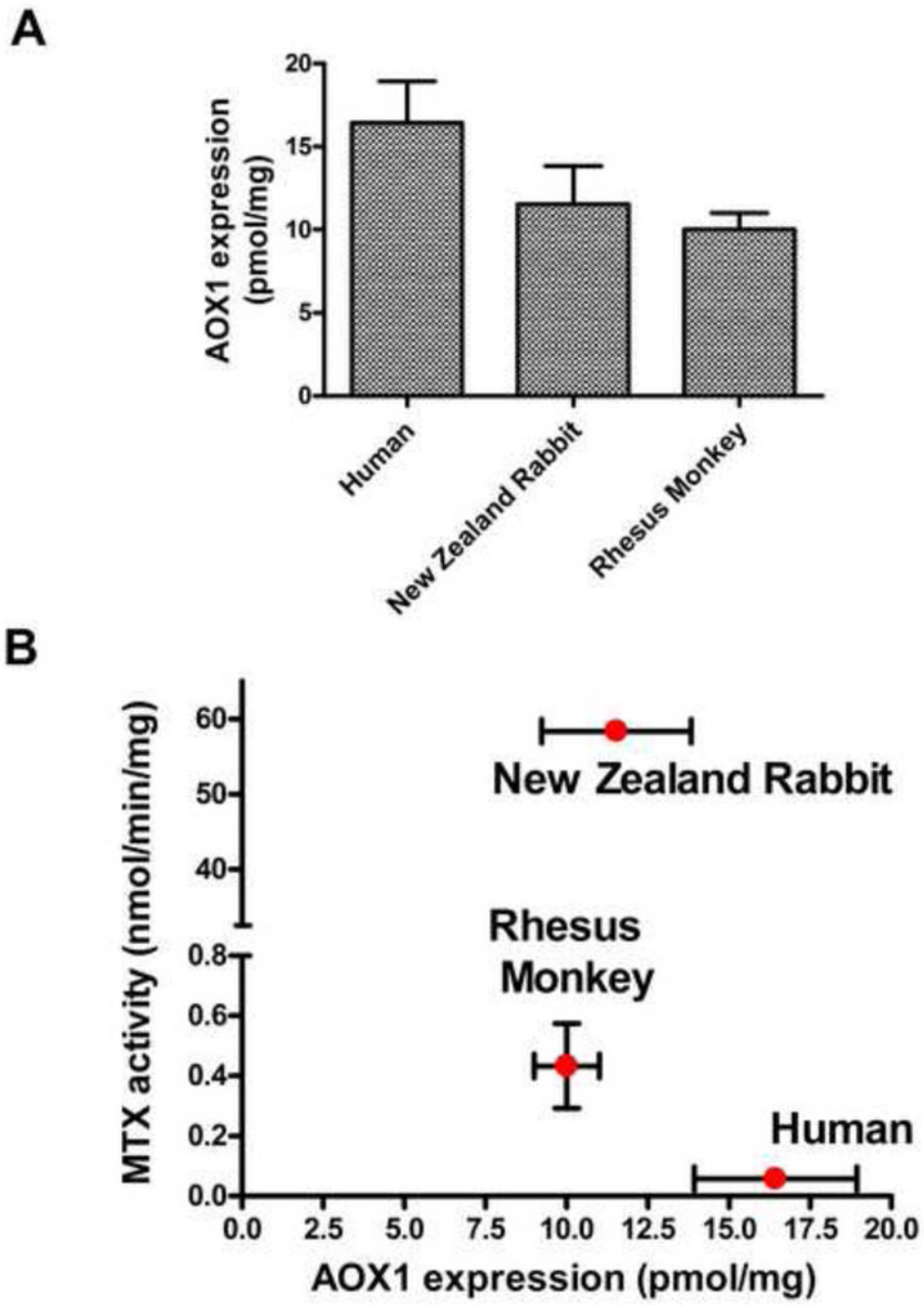

Fig. 1.

Absolute expression levels of AOX1 in the liver cytosol of Human, New Zealand Rabbit and Rhesus Monkey (A). Data were an average of triplicate experiments ± SEM. Correlation of absolute AOX1 levels with cytosolic methotrexate hydroxylation activity represented by V_{max} (B).

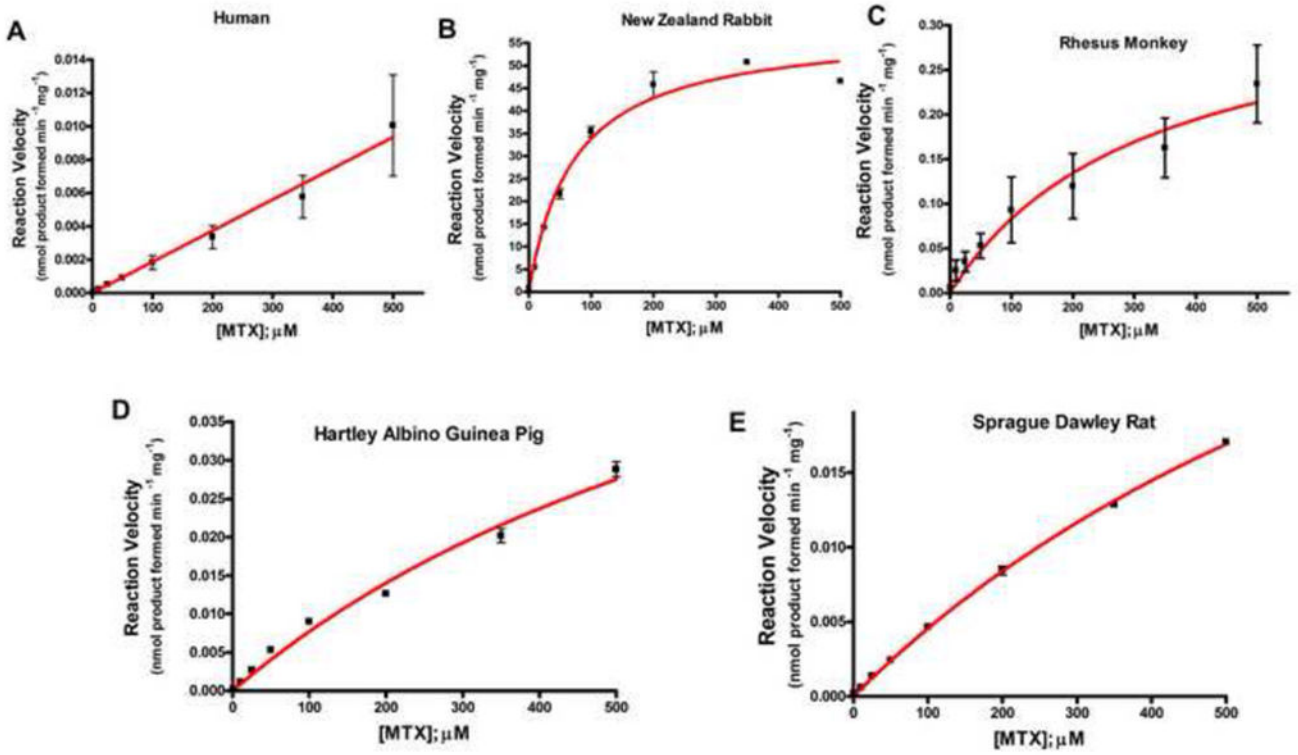

Fig. 2.

Kinetics of 7-hydroxymethotrexate formation in the liver cytosol from Human (A), New Zealand Rabbit (B), Rhesus Monkey (C), Hartley Albino Guinea Pig (D), Sprague Dawley Rat (E). Data were obtained from 3 experimental replicates ± SEM and were fitted to Michaelis-Menten equation.

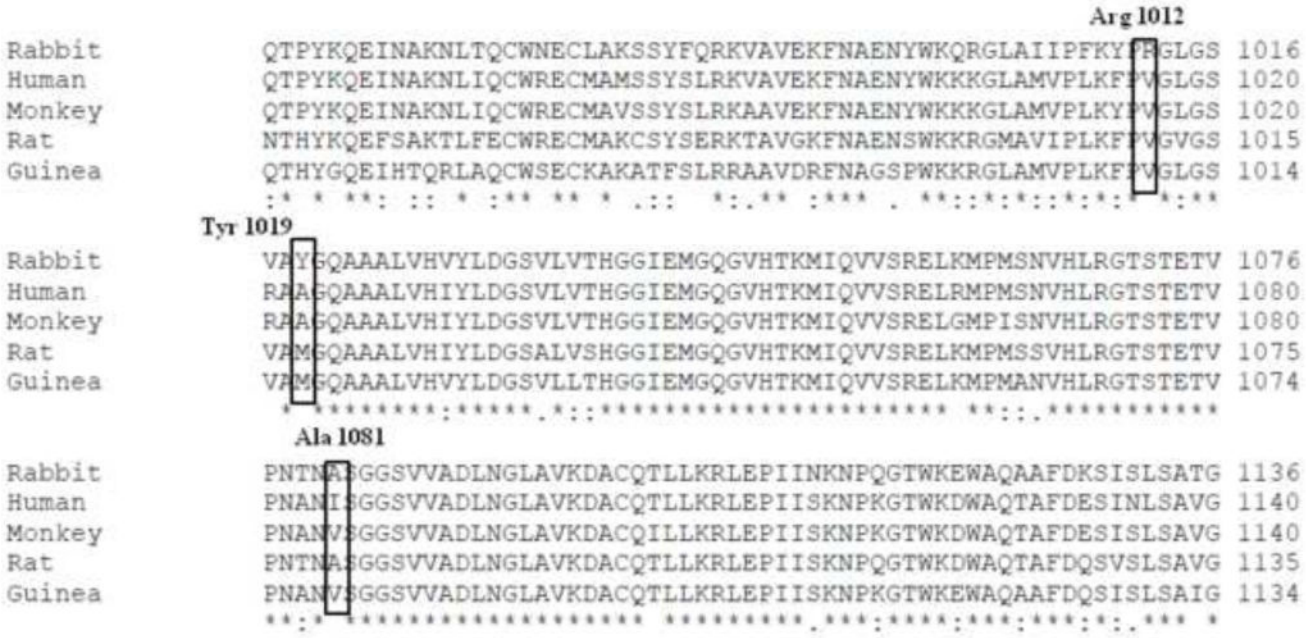

Fig. 3.

Alignment of amino acid sequences of AOX1 of rabbit, human, rhesus monkey, rat and guinea pig. Three amino acid residue differences between human and rabbit AOX1 active site are displayed in boxes with the amino acid number.

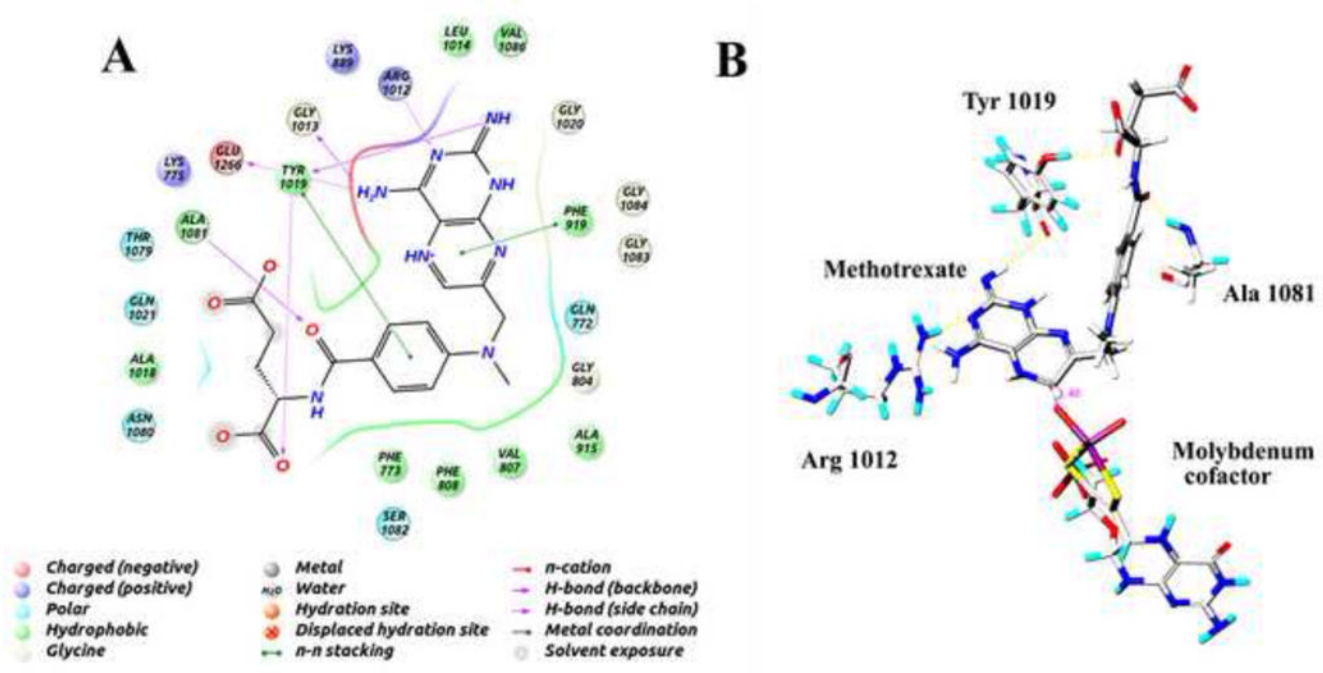

Fig. 4.

(A) Two dimensional ligand interaction diagram showing the binding environment around Methotrexate in the substrate binding pocket of rabbit AOX1. (B) AOX1 residues Arg1012, Tyr1019, Ala1081 (Corresponding to human 1016, 1023, and 1085) which are the only predicted differences in the active-site between human and rabbit enzymes with the tetrahedral intermediate complex of methotrexate and AOX1.

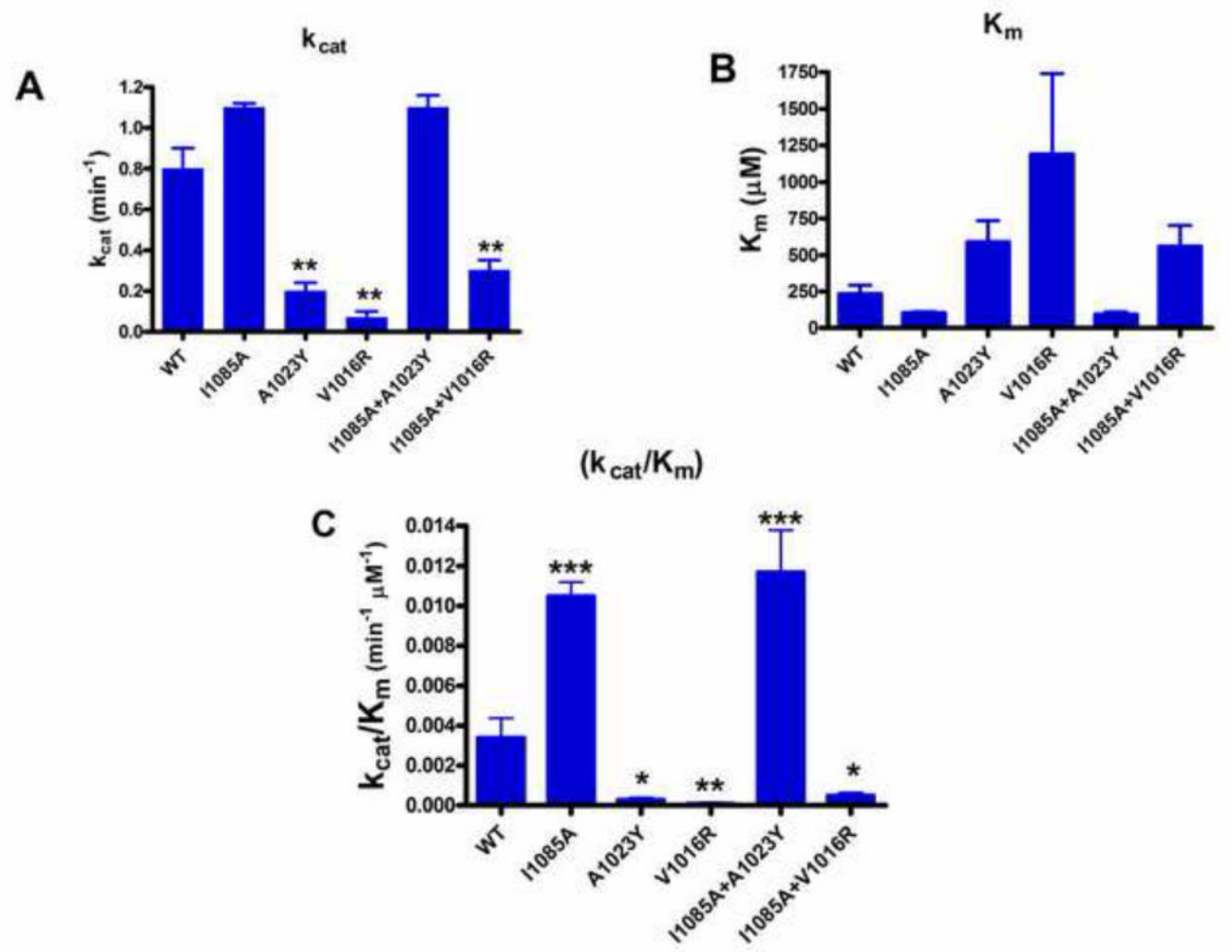

Fig. 5.

Kinetic constants k_{cat} (A), K_m (B), k_{cat}/K_m (C) for methotrexate hydroxylation by human AOX1 mutants. WT stands for wild type human AOX1 enzyme. Also shown are human I1085A, A1023Y and V1016R mutants. Data are an average of triplicate determinations ± SEM. *p < 0.05; **p < 0.01; ***p <0.001

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

Table 2

Primer sets used for preparation of human aldehyde oxidase 1 mutants

Amino acid substitution		Primer sequences
I1085A	Forward	5′ ctgtccctaatgcaaatgcctctggaggttctgtgg 3′
	Reverse	5′ ccacagaacctccagaggcatttgcattagggacag 3′
A1023Y	Forward	5′-gccttggctcacgtgcttatggtcaggctgct-3′
	Reverse	5′-agcagcctgaccataagcacgtgagccaaggc-3′
V1016R	Forward	5′-ggtccccctgaagtttcctcgtggccttggc-3′
	Reverse	5′-gccaaggccacgaggaaacttcagggggacc-3′

Table 3

Kinetic parameters for Methotrexate hydroxylation in liver cytosol of human, rabbit, monkey, rat and guinea pig.

Species	V_{max} (pmol/min/mg)	K_m (μM)	k_{cat} (min^{-1})*	In vitro CL_{int} AOX1 ($min^{-1}mM^{-1}$)
Rabbit	58400 ± 230	72 ± 9.5	5065	50650
Monkey	350 ± 70	323 ±123	35	116
Guinea Pig	70 ± 10	889 ± 214	ND[1]	ND[1]
Rat	50 ± 3	1050 ± 94	ND[1]	ND[1]
Human	40 ± 7	2792 ± 505	2.4	0.9

*k_{cat} was calculated using information from figure 1A. Data are an average of triplicate experiments ± SEM.

ND[1]: The sequence of the isotopically labelled peptide standard used for quantitation was not conserved in AOX1 of the rodent species used in this study and hence it was not possible to quantitate AOX1 levels in these species using the absolute quantitation assay described in the ‘Materials and Methods’ section.