Evaluation of vanillin as a probe drug for aldehyde oxidase and phenotyping for its activity in a Western Indian Cohort

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Abstract:
BACKGROUND: Aldehyde oxidase (AO), a molybdoflavoenzyme, is emerging as a key player in drug discovery and metabolism. Despite having several known substrates, there are no validated probes reported for studying the activity of AO in vivo. Vanillin (4-hydroxy 3-methoxy benzaldehyde) is an excellent substrate of AO, in vitro. In the present study, vanillin has been validated as an in vivo probe for AO. Subsequently, a phenotyping study was carried out using vanillin in a subset of Indian population with 100 human volunteers.

METHODS: For the purposes of in vitro probe validation, initially the metabolism of vanillin was characterized in partially purified guinea pig AO fraction. Further, vanillin was incubated with partially purified xanthine oxidase fraction and AO fractions, and liver microsomes obtained from different species (in presence and absence of specific inhibitors). For the phenotyping study, an oral dose of 500 mg of vanillin was administered to the participants in the study and cumulative urine samples were obtained up to 8 h after giving the dose. The samples were analyzed by high-performance liquid chromatography and metabolic ratios were calculated as peak area ratio of vanillic acid/vanillin.

RESULTS: (a) The results of the in vitro validation studies clearly indicated that vanillin is preferentially metabolized by AO. (b) Normal distribution tests and probit analysis revealed that AO activity was not normally distributed and that 73.72% of the participants were fast metabolizers, 24.28% intermediate metabolizers, and 2% were slow metabolizers.

CONCLUSIONS: Data of the phenotyping study suggest the existence of AO polymorphism, in a Western Indian cohort.

Keywords: Phenotyping, poor metabolizer, vanillin, Western Indian

Introduction

Aldehyde oxidase (AO) and another related enzyme xanthine oxidoreductase/xanthine oxidase (XO) are the members belonging to the family of “molybdenum hydroxylases” and are known to catalyze the metabolism of both compounds present endogenously and also compounds foreign to the body, namely xenobiotics. These are cytosolic enzymes that find wide distribution in the animal kingdom and are members of the non-cytochrome P-450 family of enzymes. Both these enzymes require a flavin cofactor for catalytic activity. Although, the two enzymes are found to have a high degree of homology with respect to their amino acid sequences, they exhibit diverse substrate affinities. XO is responsible for the

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metabolism of drugs such as doxorubicin, mitomycin-C, 6-thioguanine, and mandione, while drugs such as brimonidine, carbarzeran, famciclovir, and zaleplon, are some of the known substrates for AO.[3,5]

For xenobiotics metabolized by the CYP450s, there are well-established methods for prospective estimation of parameters such as clearance, inter-patient variability, and drug-drug interactions. However, methods for such pharmacokinetic predictions with respect to compounds metabolized by non cytochrome enzymes (one of which is AO) are not yet well established.[1,6,7] Regardless of the metabolic pathway, there are two key requirements for such predictions – (a) identifying a suitable probe drug; one that has an enzymatic biotransformation pathway that is exclusively catalyzed by the non cytochrome enzyme, to assess the in vitro activity of the enzyme and (b) subsequent generation of phenotypic and genotypic data, with respect to the enzyme, in healthy participants.

Relative to AO, XO is a better studied enzyme and phenotyping and genotyping data for XO is currently available for a few ethnic populations that include Caucasians, Ethiopians, Japanese, and the Greeks.[8-11] The realization of the role played by human AO in xenobiotic metabolism is more recent and rapidly growing. Thus, identification of a suitable probe drug for AO and subsequently generating phenotype and genotype data for AO would be of considerable interest.

The well-known flavoring agent, vanillin, is a natural product which is obtained from vanilla bean and is a “Generally Regarded as Safe or GRAS” molecule.[12] It has been found to be an excellent substrate of AO in vitro, in guinea pig AO containing subcellular fractions[13] which is very similar to human liver AO.[14] Based on this knowledge, the present study was carried out with the dual objectives of validating vanillin as an AO probe drug/substrate in an in vitro model as also subsequently using it for phenotyping of AO activity in normal, healthy, human participants.

Methods

The study was carried out in two parts: (I) The in vitro probe validation study and (II) Phenotyping study in normal, healthy, human participants.

In vitro probe validation study

We first studied the in vitro metabolic conversion of vanillin to vanillic acid to establish that vanillin was exclusively metabolized to vanillic acid by AO.

Materials used in the experiments

Vanillin was obtained from Himedia and Vanillic acid and allopurinol from Sigma Aldrich, USA. Raloxifene was obtained from Clesynth Laboratories Ltd, and nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (β-NADPH) from Sisco Research Laboratories Ltd. Human, dog, and monkey liver microsomes and human liver cytosol were obtained from Xenotech LLC, USA. The AO fraction was obtained from the livers of guinea pigs, and the XO fraction was isolated from hepatic tissue of Wistar rats, by partial purification using ammonium sulfate precipitation as described by Kadam and Iyer.[15,16] Rat liver microsomes were prepared as reported by Walawalkar et al.[17] Animal ethics committee permission was obtained.

Methods

Liquiixx kit was used to estimate the protein content of the isolated enzyme fractions and microsomes by the Biuret method.

This part of the study included the following experiments. Note that, unless otherwise specified, all enzyme incubations were done in potassium phosphate buffer (0.05 M, pH 7.4) in a volume of 600 µl and temperature of 37°C. Furthermore, during sample preparation, all centrifugations steps were done at 8000 × g for 10 min.

Evaluating metabolism of vanillin to vanillic acid (the primary metabolite of vanillin) by aldehyde oxidase using aldehyde oxidase rich hepatic cytosolic fraction obtained from guinea pigs

This was conducted by incubating vanillin in partially purified guinea pig AO fraction, and establishing both $K_m$ and $V_{max}$ enzyme kinetic parameters for the conversion of vanillin to vanillic acid. Briefly, $V_{max}$ is the theoretical maximum initial velocity of the reaction when the enzyme is exposed to infinitely high concentrations of the substrate while $K_m$ (Michaelis constant) is that concentration of the substrate at which one is expected to observe half the theoretical value of $V_{max}$. (Thus, an enzyme with a high $K_m$ has a less affinity for its substrate and needs a higher concentration of substrate to achieve $V_{max}$.)

Initial experiments were carried out to establish the linearity of vanillic acid formation with different concentrations of AO protein (0.05, 0.1, 0.25, and 0.5 mg/ml protein) and varying time points (0, 2.5, 5, 10, 15, and 20 min). Post incubation, 200 µl aliquots were withdrawn, and the reactions terminated with 100 µl of 0.5 M perchloric acid containing p-nitrocatechol as the internal standard (IS, 3 µg/ml). The samples were vortexed, centrifuged, and 50 µl of supernatant was injected onto the high-performance liquid chromatography (HPLC). All incubations were done in a final volume of 1.6 ml.

For establishing $K_m$ and $V_{max}$ of vanillin (0.25, 0.5, 1, 2.5, 5, 7.5, and 10 µM) was incubated with 0.1 mg
protein/ml AO fraction for 10 min. Post incubation, the samples were processed as mentioned above.

An incubation protocol was also designed to assess the inhibition of formation of vanillic acid using an AO specific inhibitor, raloxifene. Raloxifene (at concentrations of 0, 1, 3, 10, 30, 100, 300, and 1000 nM which are based on reported IC\textsubscript{50} values in literature\cite{18} was co-incubated with vanillin (7 \textmu M) in the presence of 0.1 mg/ml protein AO fraction for 10 min. Post incubation, the samples were processed similar to that mentioned above.

Confirming lack of metabolism of vanillin to vanillic acid by xanthine oxidase
XO is also a molybdenum hydroxylase enzyme and can potentially metabolize vanillin to vanillic acid. Vanillin was incubated with partially purified rat liver XO fraction. Briefly, incubations were done in a volume of 500 \textmu l at final concentrations of vanillin (25 \textmu M), and XO fraction (0.2 mg protein/ml). Incubations were conducted for 30 min and terminated with 250 \textmu l of 0.5 M perchloric acid containing p-nitrocatechol as internal standard (IS, 3 \mu g/ml). The samples were vortexed, centrifuged, and an aliquot of the supernatant (50 \textmu l) was injected onto the HPLC.

Further, inhibition of formation of vanillic acid was also studied using allopurinol, a specific inhibitor of XO. The incubation conditions were similar to that mentioned above and contained allopurinol at a final concentration of 5 \textmu M. Post incubation, the samples were similarly processed.

Since XO rich fractions can potentially be contaminated by small amounts of AO,\cite{15,16} the inhibition of formation of vanillic acid by raloxifene was also studied in the XO fractions. The incubation conditions were identical to that mentioned earlier, except that they contained raloxifene at a final concentration of 10 \textmu M. Post incubation, the samples were similarly processed.

Confirming lack of metabolism of vanillin to vanillic acid by CYP450s
This experiment was conducted by incubating vanillin in hepatic microsomal enzyme fractions obtained from four different animal species (human, rat, dog, and monkey). Briefly, incubations were conducted in a final volume of 500 \textmu l, and at a concentration of 25 \textmu M vanillin, 0.5 mg microsomal protein/ml, and 0.6 mM NADPH. Incubations were conducted for 30 min and terminated with 250 \textmu l of 0.5 M perchloric acid containing p-nitrocatechol as internal standard (IS, 3 \mu g/ml). The samples were vortexed, centrifuged, and an aliquot of the supernatant (50 \textmu l) was injected onto the HPLC.

Since, microsomal fractions have also been reported to be contaminated by XO and AO during isolation procedures, inhibition of formation of vanillic acid in microsomes was studied in presence of both their inhibitors, allopurinol (XO) and raloxifene (AO). Separate incubations with the same conditions mentioned above and containing allopurinol (5 \mu M) and raloxifene (10 \mu M), respectively, were conducted.

It should be noted that, both vanillin (25 \mu M, K\textsubscript{m} = 7 \mu M) and inhibitors (allopurinol and raloxifene) were used at concentrations higher than their reported IC\textsubscript{50} values to detect even a fairly low affinity formation of vanillic acid. This was done to detect metabolite formation by even a minuscule amount of AO or XO contamination in XO/CYP450 enzyme preparations.

Overall, incubations, in the presence and absence of NADPH (to detect CYP450 mediated metabolism), and with and without inhibitors of XO and AO (to detect role of XO vs. AO contaminants), respectively, were conducted in microsomal preparations.

High-performance liquid chromatography analysis of vanillin and vanillic acid for in vitro assays
The analysis was carried out on Waters Alliance 2695 Separation module having a ultraviolet/visible detector at 280 nm and chromatograms were analyzed by Empower 2.0 version software. Mobile phase containing 1\% acetic acid (A) and acetonitrile (B) was used at A: B: 85:15, and a flow rate of 1 ml/min. The retention times of vanillin, vanillic acid, and p-nitrocatechol were 10.2, 6.7, and 13.5 min, respectively.

Statistical analysis
K\textsubscript{m} and V\textsubscript{max} were derived by linear regression analysis of double reciprocal plot (Lineweaver-Burk plot) and a related method of plotting, the Eadie-Hofstee plot of the velocity and substrate concentration data. IC\textsubscript{50} values for drug and metabolite were calculated from the data plotted as log inhibitor concentration on X axis and percent inhibition on the ordinate. The plotting and analysis functions in Microsoft Excel program of the Microsoft Office Suite 2007 were used.

Phenotyping study
Ethics
The study protocol was presented to and approved by the Institutional Ethics Committee. A written, informed consent was taken from all participants before the start of the dosing. The protocol approval number is EC/OA/-48/2015 and the trial is registered with the Clinical Trials Registry of India (CTRI/2015/09/006225).

Choice of vanillin as a substrate
Vanillin is a flavoring agent that is known to be GRAS. It was chosen after the in vitro studies proved its specificity for metabolism by AO.
**Study procedure**
A total of 100 normal, healthy participants (46 women and 54 men) aged between 18 and 45 years were recruited. Those with a history of drug or alcohol use 48 h before the study, vanillin allergy, or those on treatment for any chronic condition were excluded from the study. A general medical and physical examination was then carried out. Participants were asked to abstain from vanillin containing foods and beverages 24 h before the study and until the last urine sample collection. After an overnight fast, on the day of the study [Day 1], the participants were given 500 mg of vanillin in a capsule on an empty stomach and cumulative urine was collected over the next 8 h. Aliquots were kept at −80°C and later analyzed by HPLC.

**Sample preparation and high-performance liquid chromatography analysis**
This method was developed in house. Briefly, a 2 ml aliquot of each participant sample was heated in presence of 1 ml of 0.5M perchloric acid containing para-nitrophenol as internal standard, at 95°C for 5 h, to cleave the glucuronide and sulfate conjugates. At the end of 5 h, the samples were cooled to room temperature, volume made up to 5 ml with water, and centrifuged at 8000 ×g for 5 min. The supernatants were then analyzed on HPLC at 300 nm using a method as reported by Farthing *et al.* that was modified suitably. The retentions times of vanillin, vanillic acid, and p-nitrocatechol were 11.4, 9.4, and 16.4 min, respectively.

**Evaluation of phenotype**
Metabolic ratios (MR) values were calculated by taking the ratio of peak area ratios of vanillic acid/internal standard and vanillin/internal standard. MR values were tested for normality using the Kolmogorov–Smirnov (KS test), the D’Agostino and Pearson Omnibus and Shapiro–Wilk tests in Graph Pad Prism version 5.0 using the column statistics option in column analyses. Further, a probit analysis was carried out to determine the value of antimode (cutoff point) to distinguish between slow and fast metabolizers.

**Results**

**In vitro probe validation study**

**Evaluating metabolism of vanillin to vanillic acid by aldehyde oxidase in the hepatic aldehyde oxidase fraction from guinea pig**
Formation of vanillic acid was found to increase linearly with an increase in protein concentration and time. From the results of the linearity experiments, protein concentration (0.1 mg/ml) and time (10 min) were chosen for further experiments. The kinetic parameters, $K_m$ and $V_{max}$ were estimated to be 7 μM and 0.232 nmol/mg/ml, respectively. Protein concentration (0.1 mg/ml), incubation time (10 min), and vanillin concentration (7 μM) were the optimized conditions for the inhibition assay. Using these conditions, the IC$_{50}$ of raloxifene was found to be 98.62 nM. Allopurinol did not inhibit the formation of vanillic acid to any significant extent.

**Evaluating metabolism of vanillin to vanillic acid by xanthine oxidase**
On incubation of vanillin with rat hepatic XO fraction, approximately 4% conversion to vanillic acid was seen. This conversion was not significantly inhibited by allopurinol, a known inhibitor of XO, indicating that vanillic acid formation is not XO mediated. However, this conversion was significantly inhibited (up to 76%) by raloxifene, a specific inhibitor of AO.

**Evaluating metabolism of vanillin to vanillic acid by CYP450s**
Vanillic acid formation was seen in rat and monkey liver microsomes to a small extent (3%–4%) both in the incubations with and without NADPH. CYP450 catalyzed production of a metabolite necessarily requires NAPDH cofactor. Further, this conversion was not inhibited by allopurinol, Raloxifene, on the other hand, showed up to 80% inhibition of vanillic acid formation, both with and without NADPH. Human liver CYP450 microsomes showed an insignificant formation of vanillic acid. No vanillic acid formation was seen in dog liver microsomes.

The results of all *in vitro* experiments are summarized in Table 1.

**Human phenotyping studies**
A representative HPLC chromatogram of a participant urine sample is presented in Figure 1. AO phenotyping data, expressed as the MR of vanillic acid/vanillin are presented as a frequency distribution in Figure 2. MR values were not normally distributed as reflected by the *P* values of the three statistical tests; KS test (*P* = 0.027), Shapiro–Wilk test (*P* = 0.0008) and D’Agostino and Pearson Omnibus test (*P* = 0.005). Probit transformations of the log MR values resulted in nonlinear probit plots with bimodal distribution, as shown in Figures 3 and 4. Participants with MR value <9.7 (i.e., the antimode/cutoff value, log MR 0.99) were assigned as slow metabolizers and those above this value were assigned as extensive or fast metabolizers. Subjecting the data from probit analysis to Hardy–Weinberg analysis, yielded the distribution of allele frequencies expected in the sample population to be 0.1414 for the defective alleles and 0.8586 for the wild type alleles, respectively. This corresponds to 1.99% of the sample population being homozygous for the defective alleles, 24.28% of the sample population being heterozygous and 73.72% of the
Table 1: Observations on the formation of vanillic acid in different enzyme rich fractions and the effects of inhibitors raloxifene and allopurinol on the reaction

|                          | Extent of formation of vanillic acid | Extent of formation of vanillic acid in presence of raloxifene | Extent of formation of vanillic acid in presence of allopurinol |
|--------------------------|--------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| Guinea pig AO fraction   | ++++**                              | +**                                                          | ++++                                                          |
| Rat liver XO fraction    | +                                   | −**                                                          | −                                                             |
| Rat liver microsomes     | +                                   | −                                                             | +                                                             |
| Monkey liver microsomes  | +                                   | −                                                             | +                                                             |
| Dog liver microsomes     | −                                   | −                                                             | −                                                             |
| Human liver microsomes   | +                                   | −                                                             | +                                                             |

*Formation of vanillic acid with $K_m$ and $V_{max}$ of 7 µM and 0.232 nmol/mg/ml, respectively, **Inhibition of vanillic acid formation by raloxifene with $K_i=98.62$ nM, ***Zero to negligible, Formation of vanillic acid in microsomes was to the same extent both in the presence or absence of NADPH and the effect of inhibitors was the same in either case. NADPH=Nicotinamide adenine dinucleotide phosphate, AO=Aldehyde oxidase, XO=Xanthine oxidase.

Discussion

Several literature reports have shown that AO catalyzes the metabolism of drugs containing aldehydes and nitrogen heterocycles. The conversion of the anticancer
drug used to treat metastatic colorectal cancer, 5-fluoropyrimidine, to its active form (5-fluorouracil) is mediated by AO. AO is also implicated in the metabolism of antimalarials (quinine), anticancer drugs (methotrexate, 6-mercaptopurine, and cyclophosphamide), antiviral drugs (famciclovir, zidovudine AZT), antipsychotic drugs, and antiepileptic drugs (zonisamide).[20] Although the number of metabolic reactions catalyzed by AO is minuscule relative to those by CYP450 superfamily, AO has evolved as an important enzyme involved in drug metabolism in the past few years. The primary reason for this is the paradigm shift in drug discovery and development. Pharmaceutical corporations have avoided NCEs whose metabolism is CYP450 mediated, with the goal of attenuating CYP450-mediated drug-drug interaction potential and population differences in the clearance of drugs due to phenotypic/genetic differences in CYP450 alleles. As a result, molecules that are metabolized by non-CYP450 enzymes, for example, AO and XO, are increasingly emerging as leads.[1,2,4]

Several studies have reported in vitro variability in the levels of AO, similar to that observed for other drug metabolizing enzymes that may result in clinical pharmacokinetic variability of drugs metabolized predominantly by AO. Liver cytosolic fractions prepared from 13 Caucasian livers showed a 16.6 and 2.75-fold variation in intrinsic clearances, when assayed with N-[2′-dimethylamino) ethyl] acridine-4-carboxamide and benzaldehyde as substrates, respectively.[21] This was partially attributed to lability of AO activity during processes of homogenization and storage or inter-individual variability. Further, inter-individual variability may be due to the differences in AO gene expression or presence of genetic polymorphism. Similarly, conversion of methotrexate to 7-hydroxy methotrexate, determined in six human liver cytosol samples showed a high variability (48-fold). Since 7-hydroxy methotrexate is cytotoxic and is pharmacologically active, it has been suggested that this variation should be taken into consideration while prescribing methotrexate.[23] Variability in PK parameters of AO cleared drugs has also been reported and extent of variability seems to depend on the substrate in question.[22]

In order to better predict the pharmacokinetic attributes and clinical variability of NCEs/drugs metabolized by AO, one crucial fragment of information is the quantitative understanding of the potential inter-individual variability in the AO-mediated metabolism of drugs due to genetic polymorphisms in AO.[21] One reported, genotyping study in an Italian population has indicated a potential for differences in human AO activity in different individuals containing some of the detected SNPs, indicative of variant alleles.[22] In contrast, a phenotyping study for AO enzyme using a probe substrate has yet not been reported in the literature, to the best of our knowledge.

For the generation of phenotyping data, it is necessary to first evaluate a substrate probe’s utility based on in vitro studies using suitable in vitro drug metabolism models and consequently validate the substrate probe. Vanillin (4-hydroxy 3-methoxy benzaldehyde), is a flavoring agent procured from the bean of vanilla. Vanillin is established as a good substrate of AO in several animal species in vitro. We chose the guinea pig AO fractions that were partially purified, as guinea pig AO is reported to behave similar to human liver AO.[14] The choice of validation parameters was based on the steps outlined by Pelkonen and coworkers.[24]

The metabolism of vanillin was first elucidated in guinea pig AO fraction and kinetic parameters were established. Further, the lack of metabolism of vanillin by XO fraction and liver microsomes was studied to establish the selectivity of AO towards vanillin. In this regard, although conversion of vanillin to vanillic acid was observed in the XO fraction, the reaction was not inhibited by allopurinol, a specific inhibitor of XO. However, production of vanillic acid was strongly inhibited by raloxifene, an AO inhibitor. This indicated that the XO fraction was possibly contaminated by small amount of AO due to the similar procedures involved in their isolation, yielding the observed results.

The results of the experiments in microsomes of rat, monkey and human, with and without NADPH cofactor and/or specific inhibitors, revealed similar rates of low level conversion of vanillin to vanillic acid, irrespective of whether NADPH was present or absent in the incubation. The conversion in the absence of the cofactor NADPH indicates that the production of vanillin is not CYP-mediated and supports the involvement of a non-CYP enzyme. Microsomal preparations from commercial sources have been reported to be contaminated with cytosolic enzymes.[25] This explains the observation of vanillic acid formation in the liver microsomes, since CYP450s have not been reported, till date, to be involved in the biotransformation of vanillin to vanillic acid. In addition, since formation of vanillic acid is inhibited by raloxifene alone, the conversion observed is due to AO contamination, akin to that observed in the XO fractions. The notable exception was that dog liver microsomes did not show any observable production of vanillic acid, both in presence or absence of NADPH. Importantly, dogs have been reported to be devoid of AO activity.[14] Hence, this observation further validates the fact that metabolism of vanillin to vanillic acid in microsomes is probably due to AO contamination of microsomal samples, being seen in all microsomal samples, except those obtained from AO deficient dogs.
Overall, guinea pig AO fractions showed facile conversion of vanillin to vanillic acid. Rat liver XO fraction and microsomes from all species, except dog showed much lower extent of formation of vanillic acid. In all cases, vanillic acid production was inhibited by raloxifene alone while allopurinol did not inhibit this reaction. Thus, the results of the probe validation studies confirmed that AO alone is responsible for the bioconversion of vanillin to vanillic acid.

Vanillin was therefore used as validated substrate probe in this phenotyping study on 100 normal, healthy participants. Vanillin was found to be safe with none of the participants reporting any adverse events.

Hardy–Weinberg analysis yielded the distribution of allele frequencies expected in the sample population to be 0.1414 for the defective alleles and 0.8586 for the wild type alleles, respectively. Based on the probabilities of the presence of different allele combinations in population and in the absence of a gene dosing effect, 2% of the population appeared to be poor metabolizers.

Variations in AO activity can have serious clinical implications on the clearance of drugs metabolized by AO. A standard dosage of these drugs may lead to either therapeutic failure or toxicity, which may have toxicological implications or fatal consequences. This is of grave concern for drugs having a narrow therapeutic index. Genotyping data from the Italian population study have indicated a potential for differences in human AO activity in different individuals. The present phenotyping data further indicate that inter-individual differences exist in AO activity, at least in a subset of the Indian population. These evidences combined with the knowledge that the number of drugs metabolized by AO would probably rise significantly over the next few years, point to the importance of this data set, which may allow for better in vitro–in vivo correlation of pharmacokinetics of AO substrates.

**Conclusion**

Aldehyde oxidase phenotyping was done using vanillin as a probe substrate in a Western Indian population and the percent of slow metabolizers was 2%, suggesting the existence of a polymorphism in AO.

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**Conflicts of interest**

There are no conflicts of interest.

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