The metabolism of amiodarone by various CYP isoenzymes of human and rat, and the inhibitory influence of ketoconazole

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ABSTRACT – PURPOSE. To evaluate the metabolism of amiodarone (AM) to desethylamiodarone (DEA) by selected human and rat cytochrome P450, and the inhibitory effect of ketoconazole (KTZ). **METHODS.** Some important CYP isoenzymes (rat CYP1A1, 1A2, 2C6, 2C11, 2D1, 2D2, and 3A1 and human CYP1A1, 1A2, 2D6 and 3A4) were spiked with various concentrations of AM to determine the relative kinetic parameters for formation of DEA in the presence and absence of various concentrations of KTZ. **RESULTS.** The formation of DEA was observed when AM was exposed to each of the CYP tested, although the rates were varied. Human CYP1A1 followed by 3A4 had the highest intrinsic clearance (CLint) for DEA formation whereas in rat, CYP2D1 followed by CYP2C11 had the highest CLint. Human and rat CYP1A2 seemed to have the lowest CLint. At high concentrations of AM and KTZ, near those expected in vivo, significant inhibition of all isoforms except for rat CYP1A2 was observed. At lower concentration ranges of both drugs, the inhibitory constant was determined. At these levels, KTZ was found to potently inhibit human CYP1A1 and 3A4 and rat 2D2 and 1A1. **CONCLUSION.** Human CYP1A1 and 3A4 and rat CYP2D1 and 2C11 were most efficient in converting AM to DEA. For DEA formation, the *in vivo* administration of KTZ could inhibit other CYP isoforms besides CYP3A in human and rat.

INTRODUCTION

The drug of choice in the treatment of life threatening ventricular and supraventricular arrhythmias is the class III antiarrhythmic, amiodarone (AM) (1). The drug has gained this status in large part due to the Cardiac Arrhythmia Suppression Trials (CAST I and CAST II) which showed that it caused either a decrease or no change in mortality in comparison to other class I agents (2,3). Amiodarone is also preferred over another class III antiarrhythmic drug, d-sotalol which causes a number of the same adverse effects as class I drugs (4).

AM has a large volume of distribution and is distributed extensively to extra-vascular tissues such as liver and lung (5-7). The elimination of AM occurs mainly via oxidative metabolic pathways, with the CYP super-family being implicated. One of the major products of AM metabolism is desethylamiodarone (DEA), which is present in the plasma of human and rats after iv and oral doses of AM (5,7,8). The fraction of the dose of AM converted to DEA was recently estimated to be up to 64% in rat (8). Earlier reports have demonstrated the involvement of CYP3A4, 1A1, 2D6 and 2C8 in DEA formation in human whereas CYP3A1 and 1A1 were found to be involved in DEA formation in rats (9-11). Similar to the parent drug, DEA possesses antiarrhythmic activity of its own. Indeed, because it takes less time to exert its effect, DEA has been proposed to be a more advantageous treatment option than AM itself (12). On the negative side, DEA has been found to be more toxic towards thyroid and lung tissues than AM (13,14).

(±)-Ketoconazole (KTZ) is an imidazole derivative used as the racemate in the treatment of both superficial and systemic fungal infections (15,16). Its antifungal effect is attributed to disruption of the fungal plasma membrane through inhibition of the cytochrome P450 (CYP)-dependent enzyme lanosterol demethylase which is responsible for ergosterol synthesis (17). In drug interaction studies KTZ is commonly used to establish the role of CYP3A4 in drug metabolism (18).

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The inhibitory effect of KTZ to CYP is believed to be facilitated by two binding events, binding of the N-3 imidazole nitrogen to the heme iron, and the lipophilic portion of KTZ to a hydrophobic region of the enzyme protein (15). Recent studies have shown that in addition to CYP3A, KTZ has an inhibitory action on other CYP isoforms of human (CYP1A1, 2C9, 2C19, 2D6 and 2E1) and rat (CYP1A1, 1A2, 2C6, and 2E1) (19-22).

The combined medicinal use of KTZ and AM can lead to a significant interaction *in vivo,* which is of clinical importance given that concentrations of AM are subject to therapeutic drug monitoring (23). In rat this interaction is characterized by a 24% increase in overall bioavailability of orally administered AM (11). On the other hand, in humans the interaction of AM with azole antifungal agents is well recognized and could lead to serious clinical side effects such as Torsades de pointes (24). It has been mostly assumed that this interaction is due to inhibition of CYP3A isoenzymes by KTZ (24). Given that KTZ was shown to inhibit AM metabolism by CYP1A1, this cannot be said with certainty unless it were shown that the interaction was truly specific at the level of CYP3A4.

To better understand the comparative metabolism of AM to DEA by human and rat, herein we report on the formation of DEA by a selection of recombinant CYP isoenzymes that are commonly involved in the metabolism of xenobiotics. Knowledge of the metabolism by the rat is important due to its common use as an animal model for understanding AM pharmacokinetics and dynamics. In addition, due to previous works indicating that KTZ was not a specific inhibitor of CYP3A in rat, we also examined its ability to inhibit the formation of DEA from AM by the each of these selected CYP isoforms**.**

MATERIALS AND METHODS

Chemicals

Amiodarone HCl, ethopropazine HCl, and nicotinamide adenine dinucleotide phosphate tetrasodium (NADPH) were obtained from Sigma (St Louis, MO, USA). Desethylamiodarone (DEA) was obtained as a kind gift from Wyeth Ayerst (Research Monmouth Junction, NJ). Methanol, acetonitrile, hexane (all HPLC grade), triethylamine and sulfuric acid were purchased from EM Scientific (Gibbstawn, NJ). Potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, potassium chloride, magnesium chloride hexahydrate, sucrose, and calcium chloride dihydrate (all analytical grade) were obtained from BDH (Toronto, ON, Canada). Supersomes expressing rat CYP1A1, 1A2, 2C6, 2C11, 2D1 and 2D2 and human CYP1A1, 1A2, 2D6 and 3A4 with supplementation of CYP reductase were purchased from BD Gentest (Woburn, MA). Stock solutions of amiodarone (13.2 mg/mL) or KTZ (1.24 mg/mL) were made in methanol.

Metabolism of AM to DEA by CYP

The formation kinetics of DEA were characterized when AM was exposed to human CYP1A1, 1A2, 3A4 and 2D6 and rat CYP1A1, 1A2, 2C6, 2C11, 2D1, 2D2, and 3A1 isoforms. Each 0.5 mL incubate contained 20 pmol/mL of CYP isoform and 5 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer (pH=7.4). To incubations, AM HCl was added to provide for nominal concentrations of 0.5, 2, 5, 10, 20, 40, 80, 100 or 155 μM. For this, AM HCl was dissolved in methanol such that a total methanol concentration of 0.8% was present in each incubation mixtures The oxidative reactions were initiated with the addition of 1 mM NADPH after a 5 min pre-equilibration period. All incubations were performed at 37ºC in a shaking water bath (50 rpm) for 30 min. The reaction was stopped by adding 1.5 mL ice-cold acetonitrile. Under these conditions of time and protein concentration the increase in DEA formation rates were linear based on preliminary time- and CYP content-linearity studies. Samples were kept at -20º C until assayed for AM and DEA.

Inhibition of DEA formation by ketoconazole

To study the inhibitory effect of KTZ on AM metabolism incubations were constituted as above, except that methanol concentrations were 1.6% in AM or AM+KTZ incubates. Increasing the methanol concentration to 1.6% from 0.8% had no effect on the formation rates of DEA by any of the recombinant microsomal preparations. It has been shown previously that methanol concentrations up to 1 to 3% did not affect the inhibitory potency of

KTZ on midazolam 1-hydroxylation activity by human liver microsomes (25).

The inhibitory effect of KTZ was examined using two approaches. A single concentration percentage of inhibition was carried out using 18.8 μM of KTZ and 34.5 μM of AM in the presence of the same rat and human CYP used above. For each CYP the determinations were done in triplicate for AM and AM+KTZ incubations. These high concentrations of AM and KTZ were chosen to mimic hepatic levels *in vivo* (5,26,27). The ki of inhibition of DEA formation by KTZ was also assessed using the recombinant CYP isoenzymes with lower concentrations of drug and inhibitor. For this purpose nominal KTZ concentrations were 0, 0.025, 0.04, 0.05, 0.1, 0.16, 0.5, 1, 2.5 or 5 μM with AM concentrations of 0.5, 1, 2, 4, 8 or 20 μM. Incubations were performed in replicates of 2-3 on each day for each incubate concentration of AM and KTZ.

For rat CYP3A1, the percent disappearances of AM and DEA in the presence and absence of KTZ were compared. 1 μM of AM and DEA were incubated with CYP3A1 and with various concentrations of KTZ (0, 50, 500, 5000 nM) in incubation mixtures as described above. Incubations containing all reaction component except for CYP3A1 were used as controls to provide the initial concentrations of AM and DEA. Concentrations of AM and DEA remaining were determined. Additionally in the AM-spiked samples, the difference between the initial amount of AM and the molar sum of AM+DEA measured were determined. This was assumed to represent other metabolites of AM.

Assay

After incubation, each sample was kept at -20º C until assayed for AM and DEA. An HPLC method was used for analysis of AM and DEA (11,28). The assay had a validated lower limit of quantitation of 35 ng/ml for both AM and DEA based on 100 μL of rat plasma (28). Briefly, 30 μL internal standard (ethopropazine HCl 50 μg/mL) was added to each tube containing 0.5 mL microsomal incubation mixture and 1.5 mL of acetonitrile. Samples were vortex mixed for 30 s and centrifuged for 2 min at 2500 g to facilitate removal of protein. Thereafter, 7 mL of hexane was added and the mixture was vortex mixed for 45 s, then centrifuged for 3 min. The final organic layer was transferred into new tubes, evaporated to dryness, reconstituted with mobile phase and injected into the HPLC apparatus. This assay had reported intraday and interday coefficients of variation of less than 20 %. The reported intraday accuracy ranged from 84.8 % to 114% whereas, the interday accuracy ranged from 97.4 % to 103 %.

Data analysis

To determine the kinetics of DEA formation by the various microsomal preparations, Vmax and km were determined by fitting the simple Michaelis-Menten model to the DEA formation rate vs. AM concentration data using non-linear regression analysis (29). To judge the goodness of fit, Akaike Information Criteron, sum of least squares analysis and correlation coefficient were used. The Vmax and km were thus determined. The intrinsic clearance (CLint) for AM metabolism to DEA was calculated by determining the quotient of Vmax to km.

In the inhibition studies which used large concentrations of AM and KTZ, the effect of KTZ on DEA formation was expressed as percentage of inhibition. The ki values were determined from the incubations with lower concentrations of AM and KTZ using a two step process (30). After assay of the samples, the formation data for DEA were subjected to Lineweaver-Burke analysis. Linear regression analysis of the 1/DEA formation velocity to 1/AM concentrations yielded slopes that represented the km to Vmax ratios for each KTZ concentration. Following this, secondary plots of km/Vmax vs. KTZ concentration were constructed. These plots were also subjected to linear regression analysis, with the x-axis intercept representing the ki values.

Statistical analysis

Data were expressed as mean±SD unless otherwise indicated. One-way analysis of variance followed by Duncan's multiple range *post hoc* test, or Student's unpaired *t* test were used as appropriate to assess the significance of differences between groups. Excel (Microsoft, Redmond, WA) or Sigma Stat 2.0 (Jandel Scientific, San Rafael, CA) was used to conduct the statistical analysis of data. The level of significance was set at $p < 0.05$.

RESULTS

DEA formation

There were notable differences between the isoenzymes in the speed at which the formation of DEA occurred (Figure 1). For most CYP the formation data were well fitted to the simple Michaelis-Menten model which permitted calculation of km, Vmax and CLint (Table 1). Only rat CYP1A2 did not conform to the model (Figure 2). It showed a linear increase in DEA formation rates within the range of concentrations tested. The CLint for CYP1A2 (Table 1) was approximated by the slope of the DEA formation rate velocity plots (Figure 2).

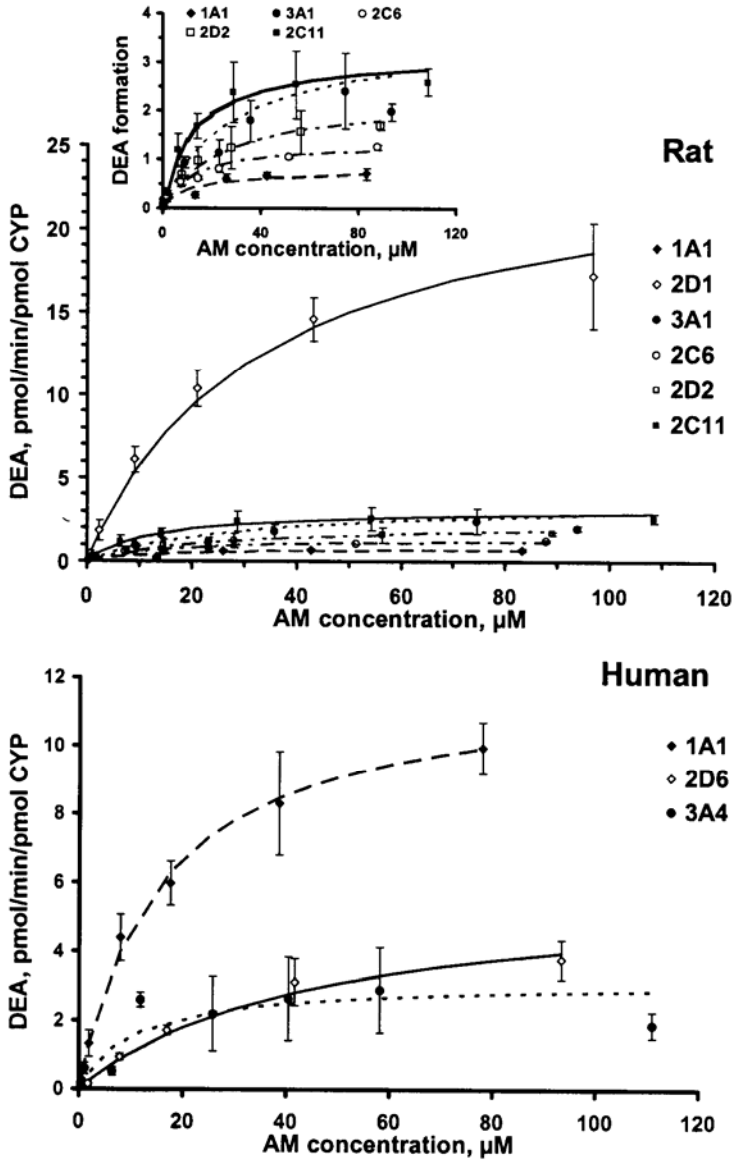

Figure 1. DEA formation rates (mean ±SD, n=3) vs. AM concentration by rat (top panel) and human CYP (lower panel). The Michaelis Menten model was fitted to the data (lines). Inset in upper panel shows the rat CYP with lower velocity.

In rat, CYP2D1 was by far the most prolific metabolizing enzyme, possessing the highest average Vmax and CLint (Table 1, Figure 1). In contrast CYP1A2 displayed the lowest CLint for formation of DEA. Although, rat CYP1A1, 2C6 and 2C11 seemed to possess higher affinity for AM, significant differences were not apparent. Kinetic analysis of DEA formation by the human CYP revealed that CYP1A1 had the highest DEA forming efficiency as indicated by its CLint. Furthermore both CYP1A1 and 3A4 had significantly higher affinity for AM, indicated by their low km values (Table 1, Figure 1). The tested rat isoforms were ranked for CLint in the following descending order; CYP2D1 > 2C11 > [2C6 = 2D2 = 3A1 = 1A1 = 1A2]. In contrast, human isoforms were ranked as follows; CYP1A1 > 3A4 > [2D6 = 1A2].

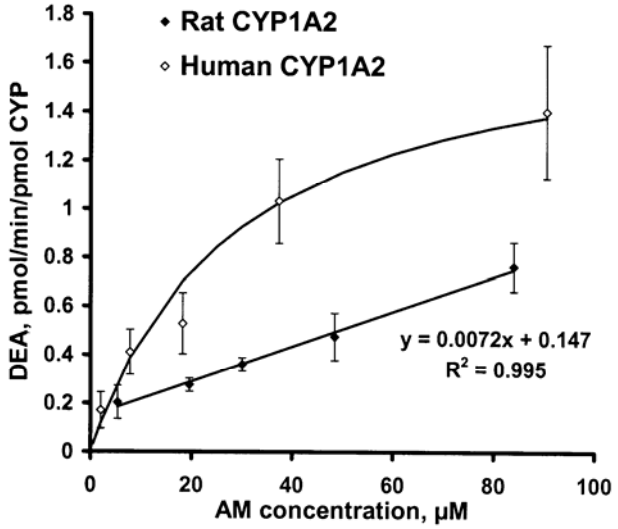

Figure 2. DEA formation rates from AM (mean ±SD, n=3) by rat CYP1A2, and human CYP1A2. The simple Michaelis-Menten model was fitted to the data for human CYP1A2 (line). Linear regression was used to get the slope of the line and estimate of CLint for rat CYP1A2.

Table 1. DEA kinetic (mean ± SD, n=3) and inhibitory constants of Vmax, km, Clint and ki in the presence of recombinant rat and human CYP isoenzymes.

CYP	Vmax, pmol/min/pmol CYP	km, µM	CLint, µL/min/pmol CYP	ki, nM
		Rat		
1A1	0.770 ± 0.0546	10.7 ± 3.03	0.0753 ± 0.0162	379
1A2	ND	ND	0.00757 ± 0.000446	ND
2C6	1.34 ± 0.124	12.2 ± 2.29	0.111 ± 0.0130	1715
2C11	3.20 ± 0.662	13.3 ± 9.05	0.308 ± 0.150	2646
2D1	25.04 ± 7.031	33.6 ± 16.5	0.802 ± 0.173	4000
2D2	2.32 ± 0.460	25.6 ± 8.51	0.0970 ± 0.0333	89.3
3A1	3.57 ± 1.24	28.6 ± 13.5	0.130 ± 0.0216	ND
		Human		
1A1	11.9 ± 1.58	15.9 ± 3.33	0.760 ± 0.0578	51.7
1A2	1.82 ± 0.550	28.9 ± 15.2	0.0685 ± 0.0186	4777
2D6	5.89 ± 1.29	45.5 ± 13.3	0.131 ± 0.0105	ND
3A4	3.15 ± 1.65	10.5 ± 3.73	0.304 ± 0.109	70.2

ND: Not determinable

Inhibition experiments

In the incubations containing high concentrations of drug and inhibitor, KTZ led to significant reductions in DEA formation rates by all of the rat isoenzymes except for CYP1A2 (Figure 3). The extent of inhibition was 86%, 76%, 58%, 88%, 55%, and 53% for CYP2D1, CYP2D2, CYP2C6, CYP2C11, CYP3A1 and CYP1A1, respectively (Figure 3). Interestingly, CYP3A1 seemed to be one of the least affected rat isoforms. Similar to rat, for the tested human isoforms, high concentrations of KTZ resulted in significant reductions in DEA formation rates by all of the tested enzymes (Figure 3). The human CYP most inhibited by KTZ were CYP1A1 and CYP3A4 (92.6% and 91.3% respectively). Human CYP2D6 (71.4 %) and CYP1A2 (50.9%) were somewhat less inhibited by KTZ.

Very good to excellent regression coefficients were obtained for most of the Lineweaver-Burke transformations (Figure 4). A concentration-dependent inhibition of DEA formation rate was observed in incubations containing rat CYP1A1, 2C6, 2C11, 2D2 and 2D1 and human CYP1A1, 1A2 and 3A4 (Figure 4). A concentration-dependent decrease in affinity (increase km) was observed for human CYP1A1 and 1A2 and rat CYP2C6 and 2D2, which is consistent with a competitive-type of inhibition. On the other hand, a concentration-dependent decrease in Vmax was observed for human CYP3A4 and rat CYP1A1, which is consistent with a noncompetitive-type of inhibition. A mixed-type of inhibition, characterized by an increase as well as a decrease in km and Vmax values respectively, was observed for rat CYP2C11 and 2D1.

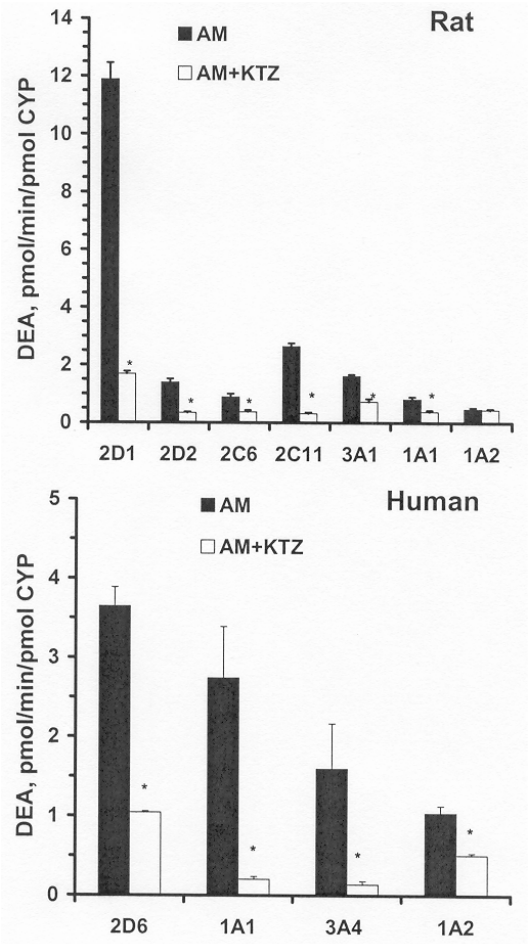

Figure 3. DEA formation rates from AM (mean±SD, n=3) in the presence and absence of 18.8 µM KTZ with 34.5 µM AM by rat CYP2D1, 2D2, 2C6, 2C11, 3A1, 1A1 and 1A2 and human CYP2D6, 1A1, 3A4, and 1A2. Incubations were run for 30 min each. *Denotes a significant difference from respective controls ($p<0.05$).

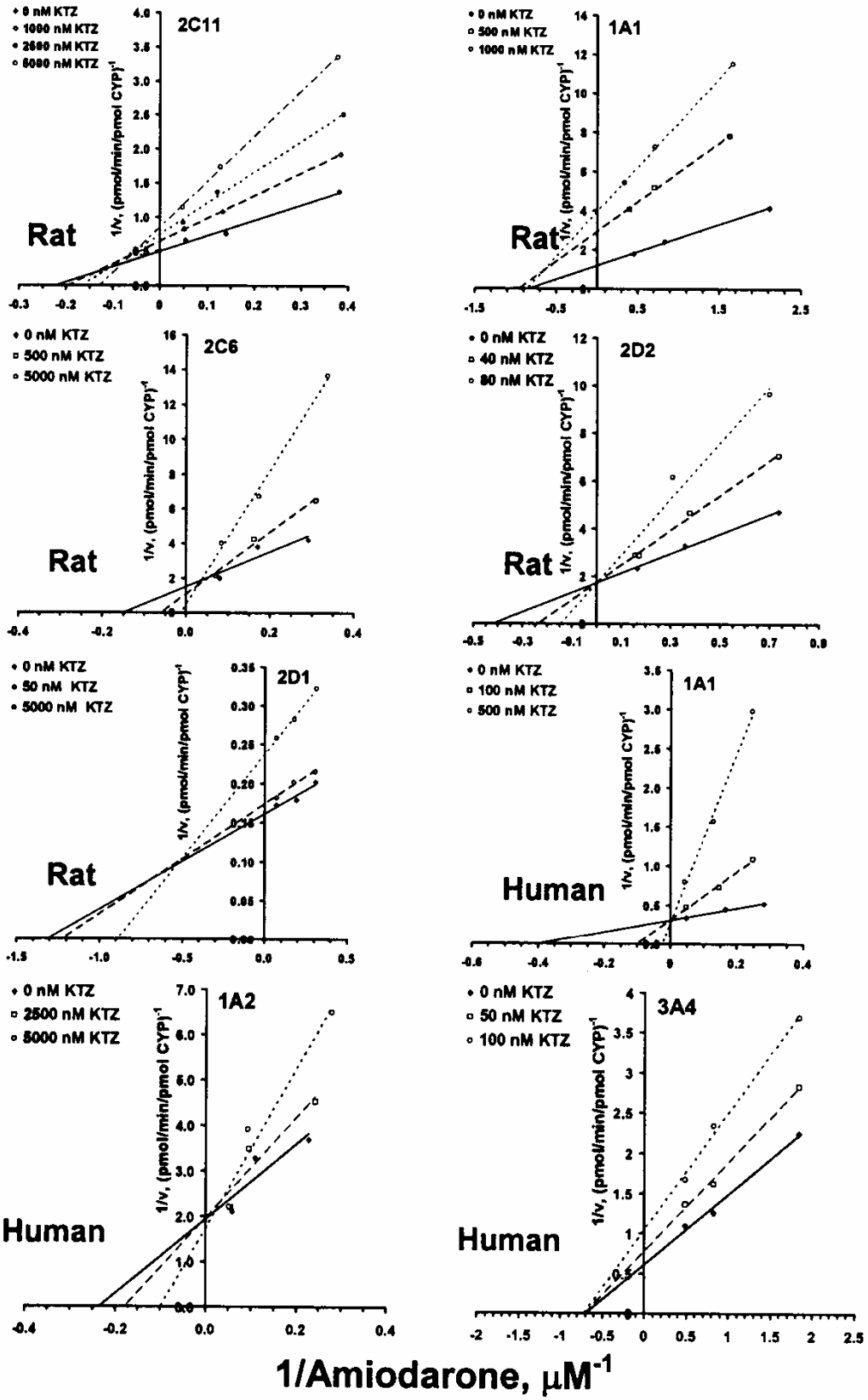

Figure 4. Lineweaver-Burke plots for rat CYP1A1, 2C6, 2C11, 2D1, and 2D2 and human CYP1A1, 1A2, and 3A4.

In the secondary plots used to determine ki (Figure 5), the mean±SD of the r^2 of slopes for different KTZ concentrations were 0.97±0.029, 0.97±0.048, 0.99±0.014, 0.90±0.10 and 0.95±0.060 for rat 2D2, 1A1, 2C11, 2D1 and 2C6, respectively. For human isoenzymes, the r^2 of Lineweaver-Burke plots were 0.95±0.091, 0.82±0.12 and 0.98±0.020 for 1A1, 1A2 and 3A4, respectively. The determined ki values were noticeably lowest for rat CYP1A1 and 2D2, and for human 1A1 and 3A4 isoforms (Figure 5, Table 1). The inhibition was much weaker for rat CYP2C6, 2C11 and 2D1 and human 1A2. Although high concentrations of KTZ caused inhibition of DEA formation by human 2D6 (Figure 3), at lower concentrations there was no apparent decrease in DEA formation.

A paradoxical increase in DEA formation rates was observed when low concentrations of KTZ were incubated with AM in CYP3A1 incubations (Figure 6). To better explore this unexpected finding, similar concentrations of AM and DEA were spiked with CYP3A1 and the decrease in concentrations of both analytes, and concentrations of DEA formed from AM, were assessed. It was noted that when directly spiked with CYP3A1, mean DEA concentrations decreased minimally and non-significantly. In contrast, the concentrations of AM were observed to significantly decrease by up to 60% or more. Increasing KTZ caused the reductions in AM concentrations to be attenuated (Figure 6). This corresponded with a reduction in the DEA formed from AM at concentrations of 5000 nM of KTZ. It was also noted that upon incubation with CYP3A1, there was approximately 28% of the initial AM unaccounted for.

DISCUSSION

The metabolism of AM to DEA has been studied in several species including human, rabbit and rat (9,11,31,32). Indeed, this biotransformation has been extensively studied in human and the involvement of CYP3A4, 2C8, 1A2 and 2D6 has already been reported, although the inhibitory effect of KTZ on these isoenzymes has not been reported (10). The activity of human CYP1A1 was not deeply investigated due to its low constitutive expression. On the other hand, although the involvement of CYP3A1, 3A2 and 1A1 in AM metabolism in rat has been reported (9,11), the possible contribution of other CYP isoforms that are abundant in rat liver has not, to date, been well studied.

Consistent with earlier reports the current results have shown that CYP3A4 possessed the highest activity amongst the constitutively expressed human isoforms. It was also of note, however, that human CYP1A1 also had a high affinity which translated into a higher CLint than CYP3A4. Despite this, because significant levels of hepatic CYP1A1 are observed under conditions of induction, CYP3A4 which constitutes ~30% of the total CYP content in human liver is expected to be the major isoform involved in AM metabolism in humans (33). The DEA formation by human CYP1A1 might however be a consideration in patients with CYP1A1 induced status, which includes patients who are smokers. Induction might explain why CYP1A1 expression is so variable in human intestine, with some specimens possessing high CYP1A1 protein and activity levels (20). In comparison to the CYP isoforms tested here, it is known that the mean km, Vmax and CLint for DEA formation by CYP2C8 are 8.6 μM, 2.3 pmol/min/pmol CYP and 0.261 μL/min/pmol CYP, respectively (10). This places the activity of CYP2C8, based on CLint, below that of both 1A1 and 3A4, respectively.

Of the tested rat isoforms, CYP2D1 showed the highest activity for DEA formation, followed by CYP2C11. the latter of which is a male-specific isoform comprising up to 54% of the total CYP content in rat liver (34). To our knowledge this is the first evidence that rat CYP2D1 and 2C11 are involved in DEA formation. Both isoforms had higher CLint values than CYP3A1 and CYP1A1 (Table 1), the roles of which biotransformation of AM to DEA was previously established (11). In rats, the expressions of genes encoding for CYP2D1, CYP3A1 and CYP2C11 are strong in the liver (35) and therefore, they could be considered as important contributors to DEA formation in rat.

In comparing human and rat CYP activities, striking differences were found in the comparison of the species-specific homologous enzymes. For example, human CYP1A1, 1A2 and 3A4 had 10-, 9-, 2-fold higher Clint than the respective rat counterparts (CYP1A1, 1A2, 3A1). Of the rat tested isoforms, only CYP2D1 showed a considerably (6.1-fold) higher CLint than human CYP2D6.

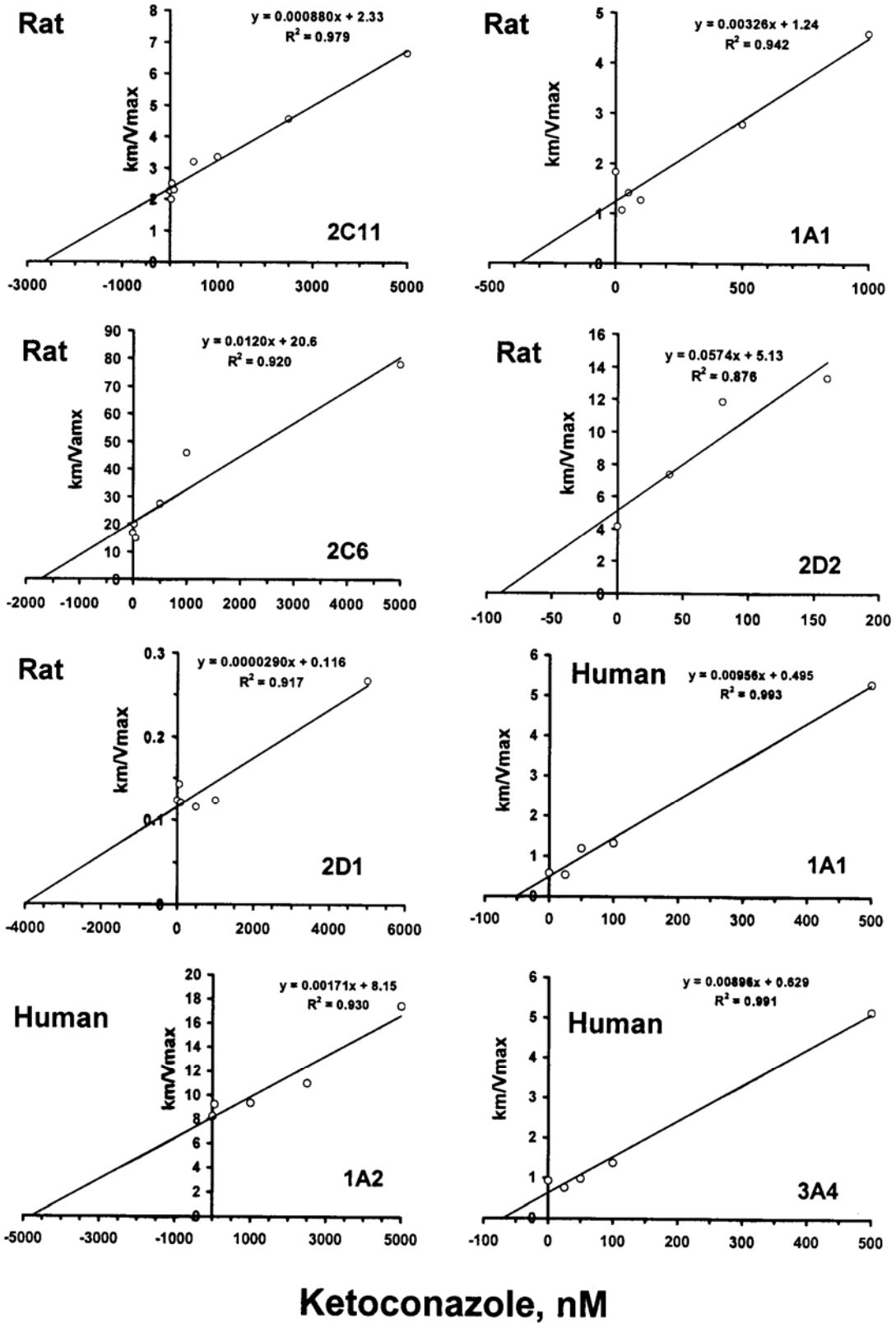

Figure 5. Secondary plots of km/Vmax (min·pmol CYP/μL) vs. KTZ concentration for A.) rat CYP1A1, 2C6, 2C11, 2D1, and 2D2 and B.) human CYP1A1, 1A2, 3A4. The ki values are provided in Table 1.

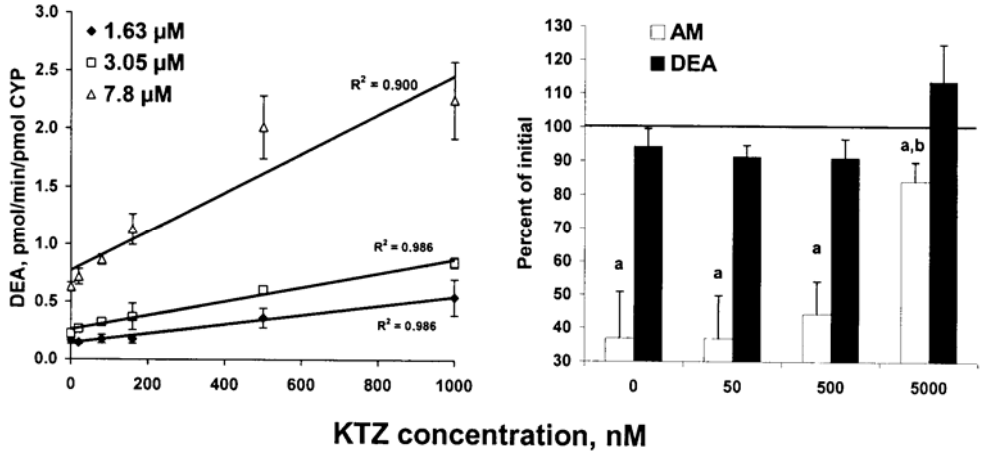

Figure 6. Formation of DEA from AM by CYP3A1. Left panel, effect of KTZ on DEA formation (mean±SD) when AM (1.63, 3.05 and 7.8 µM) was present with and without KTZ. Right panel, percent of AM or DEA remaining when 1 µM of each was incubated with CYP3A1 for 30 min, in the presence and absence of varying concentrations of KTZ. [a] denotes significant difference from amount originally added. [b] denotes significant difference from the microsomal incubation without KTZ.

Species-specific differences in isoform activity were also noticed by other investigators. For example, the oxidation of benzo[α]pyrene by human CYP1A1 was reported to be less than that of the rat CYP1A1 (36).

Ketoconazole is commonly used as an *in vitro* selective CYP3A inhibitor in humans and in rats (9,15,37-39). As such, KTZ-related *in vivo* drug interactions are commonly presumed to implicate the inhibited drugs as being metabolized by CYP3A4. There are numerous examples in the literature where this presumption is explicit, some of them being presented as late as 2007 (40,41). Given the previous findings in rat of KTZ-inhibition of CYP1A1, the current study was also designed to investigate the inhibitory selectivity of KTZ of other human and rat CYP isoforms.

For KTZ use within *in vitro* protocols it was found that although KTZ initially caused a weak accumulation of CYP3A4 protein in human hepatocytes, overall it caused inhibition of protein synthesis and inhibition of CYP3A4 marker activity suggesting a selective inhibitory effect on that isoforms (42). Thereafter, it was observed that when chemical inhibitors are used in high concentrations in *in vitro* identification of drug metabolizing enzymes, they tend to lose their selectivity. The "window of selectivity" for KTZ was estimated to be as high as 100-fold its ki value which was 15 nM (30,43). At concentrations lying within the KTZ window of selectivity (up to 500 nM), CYP1A1 was potently inhibited by KTZ (Figure 5). The ki value for CYP1A1 inhibition was near that of CYP3A4 (51.7 nM vs. 70.2 nM, respectively; Table 1). These results came in agreement with another study by Paine et al. (20) who found that CYP1A1 marker activity was inhibited by KTZ in both human gut and recombinant systems with ki value of 40 nM. The inhibitory effect of KTZ towards human CYP1A2 was very weak compared to CYP1A1 and CYP3A4. Minimal inhibitory effect for KTZ is expected as long as it is used at concentrations below 1.5 µM. However, most drug metabolism studies are conducted with high KTZ concentrations up to 100 µM (9,10), a condition that could significantly inhibit CYP1A2 and CYP2D6. For example, DEA formation from high concentrations of AM by CYP2D6 was observed to be inhibited up to 71.4% when KTZ concentrations of 18.8 µM were present (Figure 3).

In earlier reports (30,42) where KTZ inhibitory selectivity was tested against 7-ethoxyresorufin O-deethylase activity (a marker substrate for CYP1A1), an inhibitory effect was not noticed. The likely cause of this discrepancy is that all of these earlier studies were conducted in human hepatocytes or liver microsomes which in non

induced preparations express little CYP1A1 (36). Indeed, the major constitutively expressed isoform of CYP1A is 1A2 in hepatocytes, which was weakly inhibited in our studies (Figure 3).

The DEA formation facilitated by rat CYP2D2, 1A1, 2C6, 2C11 and 2D1 were each more sensitive to inhibition by KTZ than CYP3A1 (Figure 5, Table 1), which was inhibited only when a high concentration of KTZ was used (Figure 3). This finding, especially for CYP2C6, is in agreement with other reports which found that KTZ is not a selective inhibitor of CYP3A in rat (21,22). As expected, increasing concentrations of KTZ were observed to generally decrease formation of DEA. A paradoxical increase in DEA formation occurred for CYP3A1 at lower KTZ concentrations ranging from 20 to 1000 nM. Preferential inhibition of metabolism of the formed DEA by the same isoenzyme could have caused an increase in DEA formation in the presence of KTZ (44). A second explanation could be KTZ-associated inhibition of formation of other metabolite(s) of AM besides DEA, thus leading to increased amount of AM available to undergo DEA biotransformation. To address these possibilities, we conducted an experiment whereby AM and formed DEA were incubated in the presence of CYP3A1 isoenzyme. No significant metabolism or reduction in the concentration of DEA was observed when directly incubated with CYP3A1 in the absence of KTZ, which rules out the former explanation as being responsible for increased DEA formation in the presence of low concentrations of KTZ and AM. Our analytical method was not designed to measure other metabolites of AM. However, it was observed that the sum of molar-corrected [AM+DEA] formed in the AM-containing incubation was significantly less than the initial molar amount of AM added. This suggests that other metabolites of AM besides DEA are directly formed by CYP3A1, which leaves open the possibility that formation of non-DEA metabolite is preferentially inhibited by lower concentration of KTZ compared to DEA.

At the *in vivo* level, clinical dosage regimens of AM and KTZ yield hepatic concentrations of both drugs that are in the range of those used in the high concentration *in vitro* experiments. For AM, postmortem and biopsied samples of human liver showed AM concentrations ranging from 7 to 1581 μM (5,10). Similarly, in rat, high liver AM concentrations (43 and 171 μM) were present after administration of repeated high daily doses of AM (45). Similar to AM, KTZ liver concentrations were found to be at least 3.2-fold higher than those attained in plasma of rats (46). In human subjects given 600 mg KTZ, mean serum concentrations were reported to be as high as 26.3 μM (26). Assuming similar equilibration kinetics between human and rat, intrahepatic concentrations of KTZ would be expected to be significant in humans as well. Hence *in vivo*, KTZ would be expected to significantly inhibit the formation of DEA by each of the tested isoforms except for rat CYP1A2. For *in vivo* experimental results of other drugs besides AM for which KTZ is observed to be an inhibitor, it should be recognized that the involved inhibited isoenzymes may extend beyond those of the CYP3A family (Figure 3).

In conclusion, the *in vitro* metabolism of AM was species-dependent. In rat, besides CYP3A1 and 1A1 whose roles in DEA formation were already established, our results show for the first time that CYP2D1 and 2C11 are important facilitative enzymes in formation of DEA in rats. For humans, CYP1A1 and CYP3A4 were most efficient at converting AM to DEA. In the presence of high concentrations such as those expected in liver after *in vivo* administration, KTZ was observed to be a generalized inhibitor of DEA formation. At lower concentrations where ki could be identified, KTZ was found to be most potent in inhibition of human CYP1A1 and CYP3A4. In rat KTZ inhibitory potency was highest for CYP2D2 and CYP1A1.

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