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Study on the inhibitory effect of furafylline and troleandomycin in the 7-methoxyresorufin-*O*-demethylase and nifedipine oxidase activities in hepatic microsomes from four poultry species using high-performance liquid chromatography coupled with fluorescence and ultraviolet detection

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ABSTRACT

The present study reports the *in vitro* studies with furafylline and troleandomycin (TAO) as specific inhibitors of activities 7-methoxyresorufin-*O*-demethylase (MROD) and nifedipine oxidase, catalyzed by cytochrome P450 1 A2 (CYP1 A2) and 3A4 human enzymes, respectively, in hepatic microsomes of quail, duck, turkey and chicken. The results suggest that in chicken and quail the MROD activity is carried out by orthologs CYP1 A4 and 1 A5, meanwhile in duck and turkey by a CYP1 A5 ortholog. The nifedipine oxidase activity is carried out by orthologs of the CYP3A family in the four bird species. The use of furafylline and TAO significantly decreased these activities ($P < 0.05$) and suggested that the biotransformation of resorufin methyl ether (RME) may be related to more than one avian ortholog.

1. Introduction

Cytochrome P450 (CYP) are a group of enzymes responsible for the biotransformation of both endogenous and exogenous compounds. Nearly 80% of xenobiotics are metabolized by CYP1, 2 and 3 forms in humans [1]. Because of the use of pharmaceutical drugs and the exposure to pesticides and other environmental compounds, there is an increasing interest in the study of CYP enzymes able to metabolize these group of compounds. In addition, the study of CYP enzyme activity has led to explain why differential sensitivity against toxic compounds exists between species and to identify protein enzyme polymorphisms between human or animal populations [2,3].

One strategy for studying CYP enzymes is the use of prototype substrates, also called probe or marker drugs, which are compounds biotransformed specifically by certain enzymes, allowing the identification and characterization of individual enzymes [4]. At present, there are some enzyme-specific substrates used to identify the activity of some forms of human CYP enzymes [5]. In addition to the prototype substrates, the use of specific inhibitors is another important strategy to identify enzymes related to the metabolism of any substrate of interest [6]. One advantage of specific inhibitors is the possibility of determining the extent each enzyme is involved in an enzymatic reaction [7]. Different substrates and inhibitors have been used in human enzymes and in orthologs of different vertebrate groups such as birds, fish and mammals [8]. In avian species, the information on the enzymes involved in the biotransformation of different toxicants and pharmaceutical drugs is scarse [9]. To date, CYP1 A4 and 1 A5 enzymes have been identified in chickens, which are orthologous of the human CYP1 A1 and 1 A2 forms, respectively [10]. In both orthologs, 7-ethoxyresorufin-*O*-deethylase (EROD) activ-

Abbreviations: CYP, cytochrome P450; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EROD, 7-ethoxyresorufin-*O*-deethylase; HPLC, high-performance liquid chromatography; MROD, 7-methoxyresorufin-*O*-demethylase; NADP+, nicotinamide adenine dinucleotide phosphate; TAO, troleandomycin; UV, ultraviolet.

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ity was detected by using ethoxyresorufin (resorufin ethyl ether, specific human CYP1 A1 substrate) as prototype substrate, being ortholog CYP1 A4 activity higher than CYP1 A5 activity [11]. Avian CYP enzymes related to the biotransformation of xenobiotics such as aflatoxin B_1 have been identified using prototype substrates such as methoxyresorufin (resorufin methyl ether, human CYP1 A2 specific substrate), ethoxyresorufin or nifedipine (specific substrate of human CYP3A4) [12] and inhibitors of human CYP enzymes such as furafylline (1,8-dimethyl-3-(2′-furfuryl)methylxanthine; inhibitor of human CYP1 A2) and troleandomycin (TAO; inhibitor of human CYP3A4) [13–15]. Reed et al. [16] have suggested the existence of a CYP1 A5 ortholog in turkeys by using specific inhibitors. Also, inhibitors of human CYP3A subfamily have been used in avian orthologs such as erythromycin and 17-α-ethinylestradiol in turkey [17], but to the best of our knowledge, the effect of other inhibitors such as furafylline or TAO (a macrolide antibiotic that is like erythromycin, derivative of oleandomycin) on the activity of these enzymes on prototype substrates has not been determined so far. Although furafylline and TAO are specific inhibitors of human CYP enzymes [18,19], their effect on avian CYP enzymes has not been fully investigated.

In most cases, i*n vitro* systems are widely used to determine interactions of compounds with hepatic xenobiotic metabolizing enzymes. Due to CYP3A4 biotransformation of nifedipine to oxidized nifedipine and its interaction with other pharmacological compounds, high-performance liquid chromatography (HPLC) techniques using ultraviolet (UV) detection to detect this molecule have been developed. When available, in most cases detection of biotransformation products is desirable carried out by HPLC coupled to mass spectrometry [20].

In regards of this information, the principal objective of this study is to determine the expected inhibitory effect of furafylline and TAO on 7-methoxyresorufin -*O*-demethylase activity (MROD) and nifedipine oxidase in hepatic microsomes of quail (*Coturnix japonica*), duck (*Anas platyrhynchos*), turkey (*Meleagris gallopavo*) and chicken (*Gallus gallus domesticus*). Additionally, the relationship between EROD and MROD activities in ducks, turkeys and quails was determined, due to the similarity of these activities in the orthologs CYP1 A4 and 1 A5 of chicken expressed in *E. coli* [21].

2. Material and methods

2.1. Reagents

The following reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA): glucose 6-phosphate sodium salt, nicotinamide adenine dinucleotide phosphate (NADP+), glucose 6-phosphate dehydrogenase, ethylenediaminetetraacetic acid (EDTA), bicinchoninic acid, copper sulfate, sucrose, glycerol, bovine serum albumin, methoxyresorufin and TAO. Sodium chloride and magnesium chloride hexahydrate were obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA). Monobasic sodium phosphate monohydrate and sodium phosphate dibasic anhydrous were obtained from Merck (Darmstadt, Germany). The furafylline (1,8-dimethyl-3-(2′-furfuryl)methylxanthine), oxidized nifedipine (∼95%) and nifedipine were obtained from BD-Biosciences (San Jose, CA, USA). Ethoxyresorufin and resorufin sodium salt (95%) were obtained from MP Biomedicals (Solon, OH, USA). Methanol, acetonitrile, water and other solvents for the preparation of mobile phases were acquired from HPLC grade (Merck Millipore, USA).

2.2. Liver samples

Liver samples were obtained from adult animals in good health condition from turkey (*Melleagridis gallopavo*) of six weeks old, quail (*Coturnix japonica*) seven weeks old, Pekin ducks (*Anas platyrhynchos*) of seven weeks old, and chickens (*Gallus domesticus domesticus*) of seven weeks old. Age of animals is chosen because of complete expression of hepatic enzymes. Birds were sacrificed, and the livers were removed immediately; washed with PBS (20 mM phosphate, pH 7.4, 100 mM NaCl) and stored at −70 °C until the extraction of microsomes.

2.3. Microsome extraction

To obtain hepatic microsomes, samples were allowed to thaw and 2.5 *g* of liver were weighted in 50 mL centrifuge tubes kept on ice. Subsequently 10 mL of extraction buffer PBS (50 mM phosphate, pH 7.4, KCl 150 mM, disodium EDTA 0.5 mM and 250 mM sucrose) was added and then the mixture was homogenized for 30 s in a tissue homogenizer Cat X120 (Cat Scientific Inc. USA) and centrifuged at 4 °C for 30 min at 12,000 × *g* (IEC CL31R Multispeed Centrifuge, Thermo Scientific). The supernatant was then transferred (approximate volume of 10 mL) to an ultracentrifuge tube maintained on ice and centrifuged for 90 min at 100,000 × *g* (Sorval WX Ultra 100 Centrifuge, Thermo Scientific). The supernatant was discarded, and the pellet was carefully detached from the tube walls with a thin spatula and then transferred to a tissue glass homogenizer, where the pellet was suspended completely by adding 3 mL of storage buffer (phosphate 50 mM pH 7.4, EDTA 0.5 mM, 20% glycerol). The microsomal suspension was fractionated in microcentrifuge tubes and then stored at −70 °C until further analysis. An aliquot was kept for protein quantitation by the method of bicinchoninic acid modified for microplates [22].

2.4. 7-methoxyresorufin-O-demethylase, 7-ethoxyresorufin-O-deethylase and nifedipine oxidase activity

In vitro determination of enzymatic parameters of 7-methoxyresorufin-*O*-demethylase, 7-ethoxyresorufin-*O*-deethylase and nifedipine oxidase activity were carried out in 1.5 mL microcentrifuge tubes. Each incubation contained glucose 6-phosphate 5 mM NADP + 0.5 mM, glucose 6-phosphate dehydrogenase 0.5 IU, microsomal protein of 20 μg for quails, 50 μg for turkeys, 75 μg for ducks and 100 μg for chickens. Incubation buffer (phosphate 50 mM pH 7.4, $MgCl_2$ 5 mM and EDTA 0.5 mM) and 2 μL of ethoxyresorufin 0.3 to 4.8 μM, or methoxyresorufin from 0.36 to 5.88 μM or nifedipine from 4.4 to 71.0 μM nifedipine were used. All substrates dissolved in dimethyl sulfoxide (DMSO) equivalent to 0.8% of organic phase in incubation to a final volume of 250 μL [23]. Incubations were carried out for 10 min at 39 °C and reaction was stopped with 250 μL of acetonitrile at −20 °C [24]. Finally, samples were centrifuged at 15,000 x g for 10 min (Z233M-2 High Capacity Microcentrifuge, Labnet International Inc, USA) and an aliquot of the supernatant was analyzed by HPLC as described in Section 2.6. The amount of product formed was quantified using a standard calibration curve of each activity. For resorufin quantitation, solutions of 3.12, 6.25, 12.5, 25.0 and 40 nM were used. For oxidized nifedipine quantitation, solutions of 2.27, 4.53, 9.06, 18.13 and 36.25 μM were used. In both cases, standards were prepared in HPLC grade methanol and stored at −20 °C.

2.5. Inhibitor dose-response test (MROD and nifedipine oxidase inhibition activity)

Inhibition assays were performed in vitro in 1.5 mL microcentrifuge tubes. The incubation buffer containing glucose 6-phosphate 5 mM NADP + 0.5 mM, glucose-6-phosphate dehydrogenase 0.5 UI and 1 μL of methoxyresorufin 5.88 μM plus 4 μL furafylline (final concentration of the inhibitor from 192.2 μM to 12.0 μM) or 1 μL nifedipine 71 μM plus 4 μL of TAO (final con-

centration of inhibitor 61.4 μM to 3.8 μM). Both substrates and inhibitors were dissolved in DMSO corresponding to a percentage of 2.0% of organic phase [23]. Microsomal protein concentrations in the incubations were of 20 μg for quail, 50 μg for turkeys, 75 μg for ducks and 100 μg for chickens. Incubation was completed by the addition of buffer phosphate buffer 50 mM pH 7.4, $MgCl_2$ 5 mM and EDTA 0.5 mM to a final volume of 250 μL. Protein concentration was determined in preliminary tests to ensure the linearity of the reaction for a period of 10 min at 39 °C. Reactions were stopped with 250 μL of acetonitrile at −20 °C [24] and centrifuged at 15,000 × *g* for 10 min (Z233M-2 High Capacity Microcentrifuge, Labnet International Inc, USA). An aliquot of the supernatant was analyzed by HPLC as described below. The amount of product formed was quantified using a standard calibration curve. Each inhibitor was tested separately on 3 samples of microsomes from each avian species made in duplicate.

2.6. Chromatographic conditions

The amount of product formed for each enzyme activity was quantitated on a Shimadzu chromatograph (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a degasser DGU-$20A_3$, an LC-20AB pump, an autosampler SIL-20 A HT, an CTO-20 A column oven, a UV/visible detector SPD-20AV, a fluorescence detector RF-10 A XL and a CBM-20 A integrator, all controlled by computer using the L̈C Solutionsp̈rogram. The separations were carried out using a column Alltech Alltima HP C18 5 μm 150 mm × 3.0 mm in diameter (Alltech Associates Inc., Deerfield, IL, USA).

Separation of ethoxyresorufin and resorufin (EROD activity) and methoxyresorufin and resorufin (MROD activity) was performed at room temperature at a rate flow of 0.3 mL/min using a mobile phase consisting of 30% phosphate buffer (20 mM pH 7.4) and 70% methanol (isocratic mode). The analytes were detected by fluorescence at a wavelength of 530 nm excitation and 580 nm emission [25]. For each incubation, a 1:10 dilution was performed in water and a volume of 5 μL was injected into the chromatograph. Total analysis time was 15 min.

Separation of nifedipine and its oxidized product was carried out at room temperature at a flow of 0.5 mL/min with a mobile phase consisting of 32% acetonitrile and 68% water (isocratic mode). Analyses were monitored by UV detection at a wavelength of 270 nm [26]. For each incubation, a volume of 5 μL was injected, undiluted. Total analysis time was 15 min.

2.7. Database search

To investigate the presence of human CYP1 A1, 1 A2 and 3A4 avian orthologs, The National Biotechnology Information Center database [27] was consulted, where multiple alignment was performed and the percentage of identity of each human CYP against its avian orthologs was determined.

2.8. Statistical analysis

The enzymatic parameters K_M y V_{max} were determined by non-lineal regression using Marquardt method, fitting the data to the Michaelis-Menten model: $V = V_{max}\ [S] / (K_M + [S])$, where V is the enzymatic reaction velocity, [S] represents substrate concentration, V_{max} maximum reaction velocity and K_M represents Michaelis-Menten constant [28]. Statistical differences between the concentrations of the inhibitor were estimated by an ANOVA variance analysis with a level of significance $\alpha = 0.05$. Comparison of each concentration inhibitor against the control (no inhibitor was added) was probed using a Dunnet statistical test. A matrix correlation was performed between MROD, EROD, and nifedipine oxidase activities. Statistical calculations were conducted in the SAS statistical software [29].

3. Results and discussion

In regards of the detection of substrates and biotransformation products, chromatograms of substrates and products are presented in Fig. 1. Enzyme activity products were quantitated by using calibration curves, where linearity was assessed between 3.12 and 40 nM for resorufin, with a R^2 of 0.999 according to the linear model $y=(77{,}222.0 \pm 247.1)x+(9075.7 \pm 4973.4)$. In the case of oxidized nifedipine linearity was assessed between 2.27 and 36.25 μM, with a R^2 of 0.999 according to the linear model $y=(5308.5 \pm 30.1)x-(2805.7 \pm 514.8)$. The limit of detection and limit of quantification values for resorufin were of 0.62 and 1.88 nM, and 0.56 and 1.70 μM for oxidized nifedipine. Retention time presented a value of 3.785 ± 0.033 min with a for resorufin and 1.992 ± 0.037 min for oxidized nifedipine (in each case the number of injections was 10).

The values of enzymatic parameters Michaelis-Menten constant (K_M), Maximal Velocity (Vmax) and the Intrinsic Clearance (CL_{int}) in avian species are presented in Table 1. In all cases EROD, MROD and nifedipine oxidation enzyme activities are present in poultry hepatic microsomes, with a tendency of a high enzyme activity efficiency in quail microsomes, according to CLint (this enzyme parameter refers to the enzyme-mediated clearance that would occur without physiological limitations, like hepatic blood flow), in contrast to chicken, which presents lower values. The fact that prototype substrates are biotransformed enzymatically by poultry orthologs suggest that probe drugs tested on human enzymes are a potential tool in characterizing enzymes related with xenobiotic metabolism in farm animals. The ability to detect and quantitate these probe drugs and their enzymatic products, by affordable instruments like HLPC-UV-FL, make these methods available for farm animal analysis in basically equipped research laboratories.

In Table 2, Pearson coefficient values showed a positive correlation for MROD, EROD and nifedipine oxidation in most of the cases. The correlation coefficient for K_M kinetic parameter is much higher in quail and chicken for MROD and EROD activities than correlation of nifedipine oxidation with MROD or EROD, excepting duck with a Pearson coefficient of 0.88 for MROD-nifedipine oxidation correlation. In regards of Vmax, correlation coefficient is remarkable high in chicken and duck for EROD and MROD activities. Finally, CLint showed high values in quail, chicken and turkey for MROD and EROD activities. This results strongly suggest that MROD and EROD enzyme activities are carried out by a single enzyme.

Fig. 2 introduce the effect of furafylline on MROD activity, where a statistically significant reduction ($P < 0.05$) was found with respect to the control (without inhibitor) in quail and turkey microsomes. Fig. 3 shows up the effect of TAO on nifedipine oxidase activity. A significant reduction was found regarding the control in turkey, duck and chicken. In the same way as probe drugs do, specific inhibitors of human CYP enzymes appear as a useful tool for identify and characterize enzymes involved in xenobiotic metabolism. In the National Centre for Biotechnology Information database, 40 different poultry orthologous can be found. Human CYP1 A2 has a 63% identity with the chicken ortholog CYP1 A5, 62% with CYP1 A5 turkey and quail orthologs, 57% with CYP1 A4 quail and chicken orthologs and 66% with a predicted duck ortholog CYP1 A5. The alignment of the human CYP1 A1 form reports 62% of identity with the chicken and turkey CYP1 A5 orthologs, of 61% with the CYP1 A4 and 1 A5 orthologs of quail and the CYP1 A4 ortholog of chicken and of 66% with a predicted form that can be similar to an ortholog CYP1 A5 of duck. Human CYP3A4 showed 59% identity with the chicken ortholog CYP3A5, 58% with the form CYP3A37 of turkey, 60% with the form CYP3A80 of chicken and turkey, 61%

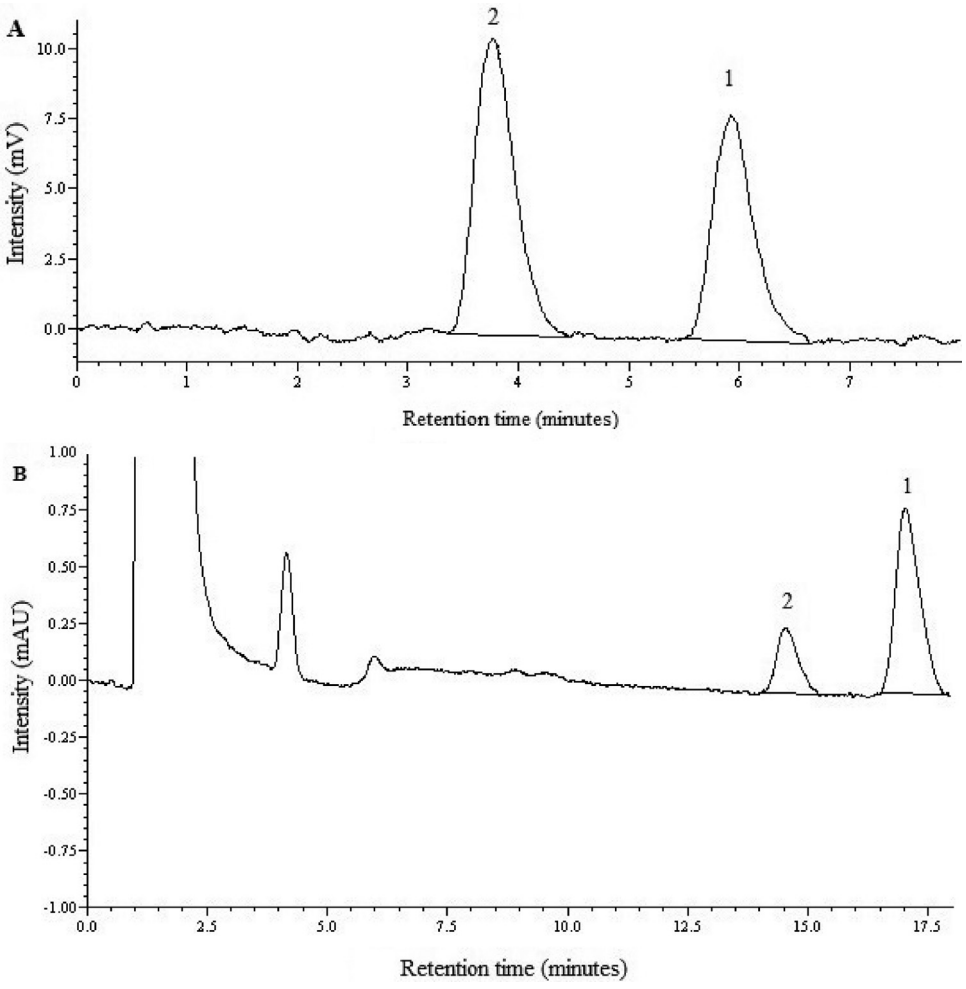

Fig. 1. Chromatographs of substrate - product couples separated by HPLC. A: 1. resorufin methyl ether (RT = 5.92 min.) and 2. resorufin (RT = 3.76 min.). Detection by fluorescence at λ_{ext}: 539 nm and λ_{em}: 580 nm respectively. B: 1. nifedipine (RT = 17.02 min.) and 2. oxidized nifedipine (14.53 min.). Detection by UV at 279 nm.

Table 1
Enzymatic kinetic parameters Michaelis-Menten constant (K_M), Vmax) and Intrinsic Clearance (CLint) of 7-methoxyresorufin *O*-demethylase (MROD), 7-ethoxyresorufin *O*-deethylase (EROD) and nifedipine oxidase enzyme activities evaluated in the four poultry species by nonlinear regression. SD: standard deviation.

Enzyme Activity	Species	KM (μM)	SD	Vmax (pmol substrate/mg protein/min.)	SD	Clint (Vmax/KM)	SD
	Quail	2.80	0.28	4.60	0.99	1.63	0.19
Nifedipine	Turkey	22.50	5.37	6.50	0.57	0.30	0.10
Oxidation	Chicken	5.15	0.49	1.35	0.07	0.26	0.01
	Duck	69.90	30.26	7.15	3.32	0.10	0.00
	Quail	0.45	0.07	0.25	0.07	0.58	0.25
7-ethoxyresorufin	Turkey	2.40	0.14	0.60	0.01	0.25	0.01
O-deethylase	Duck	0.80	0.28	0.20	0.14	0.23	0.09
	Chicken	0.35	0.07	0.02	0.01	0.06	0.01
	Duck	3.05	0.07	1.00	0.28	0.33	0.09
7-methoxyresorufin	Turkey	7.00	2.26	2.05	0.35	0.30	0.05
O-demethylase	Quail	2.40	0.71	0.65	0.07	0.29	0.11
	Chicken	0.50	0.14	0.08	0.03	0.16	0.01

with the sequences associated with forms CYP3A4, 3A8 and 3A30 of duck and of 59% with the sequences associated to the forms CYP3A9 and 3A12 of quail. So, the fact that poultry CYP ortholog sequences appeared with at least 57% of identity support the results found in this study, about the presence of CYP poultry orthologs able to biotransform human CYP probe drugs and susceptible to be affected by human CYP specific inhibitors.

In general, the biotransformation of methoxyresorufin, ethoxyresorufin and nifedipine substrates confirmed the presence of enzymatic activities 7-methoxyresorufin-*O*-demethylase, 7-ethoxyresorufin-*O*-deethylase and nifedipine oxidase in hepatic microsomes from turkey, duck, quail and chicken. These enzyme activities also suggest the presence of avian orthologs belonging to 1 A1, 1 A2 and 3A4 CYP enzyme subfamilies. The results obtained from NCBI database also support the presence of these subfamilies in poultry species. The relationship of the enzymatic parameters K_M and Vmax for the EROD and MROD activities is positive and presents high values for the coefficient of correlation (>0.75).

Table 2
Correlation matrix of enzymatic kinetic parameters Michaelis-Menten constant (K_M), Maximal Velocity (Vmax) and Intrinsic Clearance (CLint) of 7-ethoxyresorufin *O*-deethylase (EROD), 7-methoxyresorufin *O*-demethylase (MROD) and nifedipine oxidase enzyme activities evaluated in quail, duck, chicken and turkey hepatic microsomes. Pearson correlation coefficients highlighted in bold are statistically significant ($P < 0.05$).

	Quail				Duck			
		EROD	MROD	Nifedipine oxidation		EROD	MROD	Nifedipine oxidation
KM (μM)	EROD	1			EROD	1		
	MROD	**0.85**	1		MROD	0.68	1	
	Nifedipine oxidation	0.38	0.34	1	Nifedipine oxidation	0.48	**0.88**	1
	Chicken				Turkey			
		EROD	MROD	Nifedipine oxidation		EROD	MROD	Nifedipine oxidation
	EROD	1			EROD	1		
	MROD	**0.99**	1		MROD	0.44	1	
	Nifedipine oxidation	0.08	0.09	1	Nifedipine oxidation	−0.54	0.05	1
Vmax (pmol substrate/μg protein/min.)	Quail				Duck			
		EROD	MROD	Nifedipine oxidation		EROD	MROD	Nifedipine oxidation
	EROD	1			EROD	1		
	MROD	0.27	1		MROD	**0.97**	1	
	Nifedipine oxidation	0.54	0.52	1	Nifedipine oxidation	0.62	0.71	1
	Chicken				Turkey			
		EROD	MROD	Nifedipine oxidation		EROD	MROD	Nifedipine oxidation
	EROD	1			EROD	1		
	MROD	**0.99**	1		MROD	0.69	1	
	Nifedipine oxidation	0.70	0.73	1	Nifedipine oxidation	−0.23	−0.32	1
CLint (Vmax/KM)	Quail				Duck			
		EROD	MROD	Nifedipine oxidation		EROD	MROD	Nifedipine oxidation
	EROD	1			EROD	1		
	MROD	**0.80**	1		MROD	0.38	1	
	Nifedipine oxidation	0.13	0.48	1	Nifedipine oxidation	−0.33	0.26	1
	Chicken				Turkey			
		EROD	MROD	Nifedipine oxidation		EROD	MROD	Nifedipine oxidation
	EROD	1			EROD	1		
	MROD	**0.99**	1		MROD	**0.94**	1	
	Nifedipine oxidation	0.16	0.12	1	Nifedipine oxidation	0.16	0.21	1

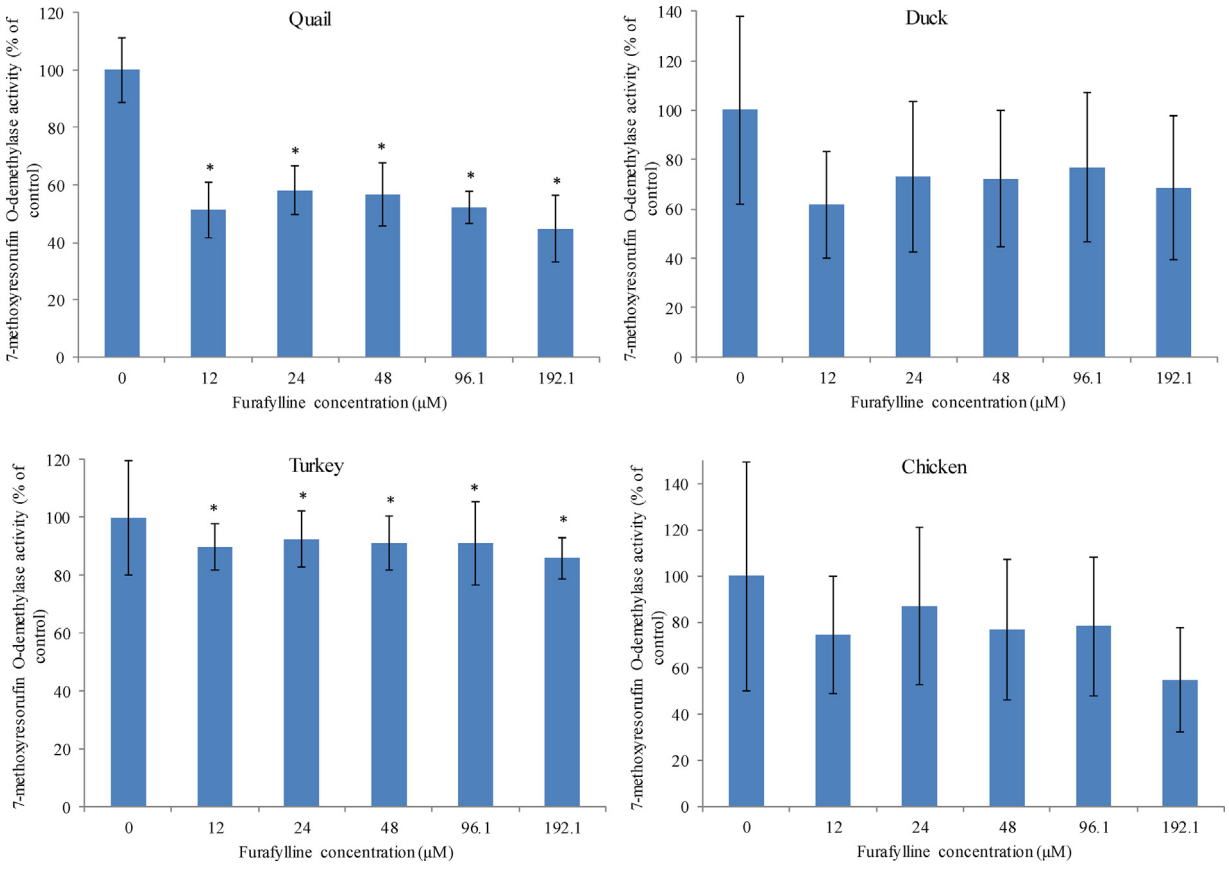

Fig. 2. Percentage of 7-methoxyresorufin-*O*-demethylase activity vs. concentration of specific inhibitor of human CYP1 A2 furafylline for each evaluated avian species. The average of three birds is presented in duplicate ± standard deviation. The highlighted concentrations with (*) presented statistically significant differences against the control ($P < 0.05$).

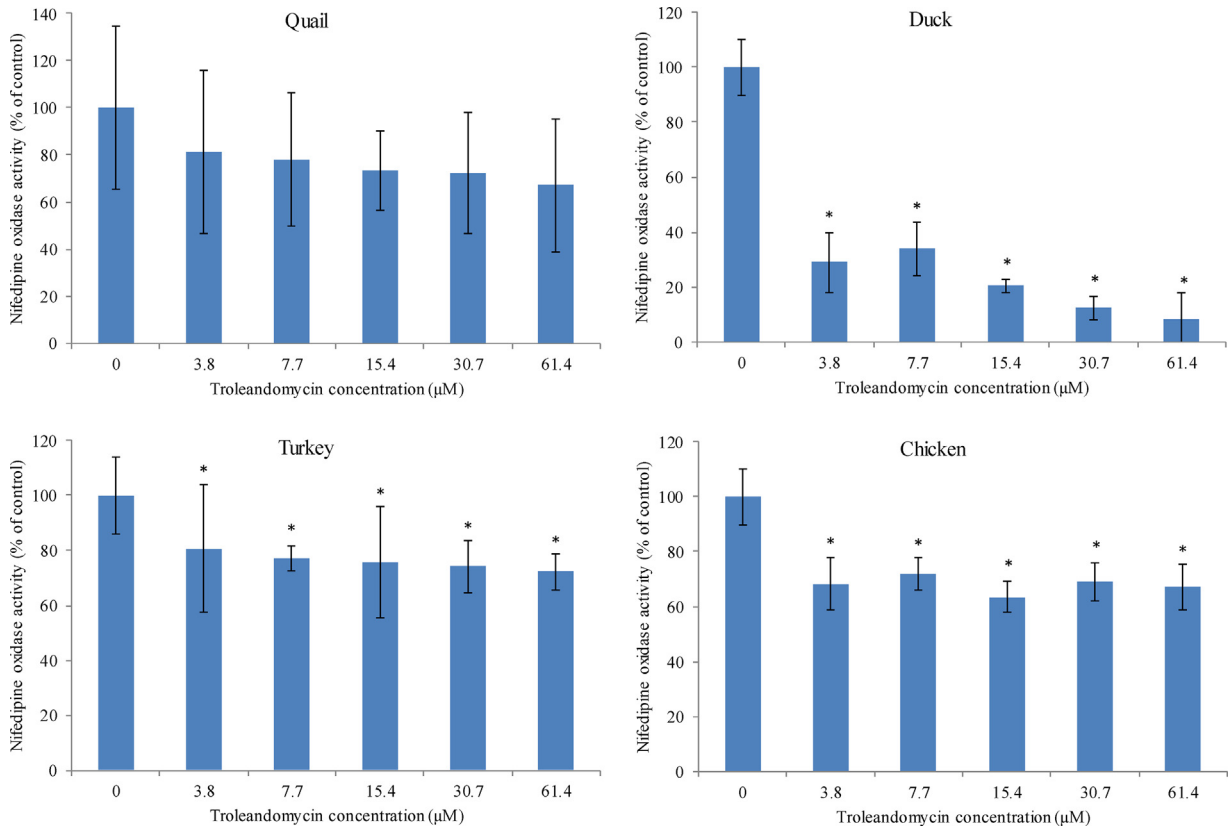

Fig. 3. Nifedipine oxidase activity percentage vs.specific inhibitor of human CYP3A4 troleandomycin for all evaluated avian species. The average of three birds is presented in duplicate ± standard deviation. The highlighted concentrations with (*) presented statistically significant differences against the control ($P < 0.05$).

Previously, Yang et al. [21] reported how CYP1 A4 and 1 A5 chicken orthologs expressed in *E. coli* presented EROD and MROD activities, showing an enzymatic activity overlap. Apparently, the same effect could be present with orthologs of turkey, duck and quail. High values for the correlation coefficient suggests that these activities are carried out by CYP1 A4 and 1 A5 avian orthologs. The use of furafylline showed a reduction of the MROD enzyme activity of about 60% in quail, while turkey reduction reaches 15%. Although an apparent inhibitory effect in duck and turkey is present, variability between individuals does not allow a statistically significant difference. With the use of TAO, a 91% inhibition of nifedipine oxidase activity in duck is observed, a 28% in turkey and 37% in chicken. In quail, an inhibitory tendency is observed but the variability between individuals also does not allow defining statistical differences. In cases where inhibition occur, 100% decrease in activity is not achieved, suggesting that other avian CYP orthologs could be involved in the biotransformation of these substrates. The correlation matrix of Table 2 shows that nifedipine oxidase activity is correlated MROD activity (Pearson correlation of 0.88) suggesting the possible relationship of a CYP3A ortholog in the biotransformation of methoxyresorufin in the duck. In humans, the CYP3A4 enzyme is the main contributor to the metabolism of pharmaceutical drugs [30], so the presence of avian CYP3A form with MROD enzymatic activities should not be discarded. In the future, the use of inhibitor cocktails that allow a 100% reduction in these enzymatic activities would led to identify the effect of different orthologs metabolizing the same substrate.

4. Conclusions

Hepatic microsomes from turkey, duck, chicken and quail contain human CYP orthologs with EROD, MROD and nifedipine oxidase activities. It is possible that the enzymes related with EROD and MROD activities are the orthologs CYP1 A4 and 1 A5 in chicken and quail, and the CYP1 A5 ortholog in turkey and duck. Regarding nifedipine oxidase activity and information from NCBI database, there is a member of the CYP3A family. Because no full inhibition was achieved in both enzymatic activities, it is presumed that other avian CYP orthologs could be related with the biotransformation of these prototype substrates, such as one of the CYP3A family. It is important to conduct future inhibition test with cocktail of inhibitors that lead to an enzymatic activity reduction close to 100%, so CYP enzymes that carry on these enzymatic activities can be identified more precisely.

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