AROMATASE INHIBITION BY AZOLE DRUGS: ASSESSING PREGNANCY RISKS

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Abstract

Human aromatase (CYP19) converts C19 androgens to aromatic C18 estrogenic steroids. Its activity is critical for pregnancy maintenance and in regulating parturition in late pregnancy. Past studies utilized placental microsome tritiated water release assay to assess drug interactions with estrogen synthesis. Data from human placental assay was compared with BD Biosciences Discovery Labware's recombinant CYP19 enzyme assay using the fluorometric substrate dibenzylfluorescein. A panel of azole antifungals was tested which may be administered to pregnant women for their potential to inhibit aromatase. Potency varied by several orders of magnitude. For example, metronidazole was consistently inactive as an inhibitor at concentrations up to 100 microM whereas ketaconazole and miconazole returned IC_{50} values of 1.5 microM and 0.04 microM, respectively. CONCLUSIONS: 1. Recombinant enzyme assay data are comparable to the human placental assay data in both SAR rank order and potency. 2. Plasma and tissue levels of some azole drugs following oral or topical administration are at or above these IC_{50} values. Therefore, some azole drugs may disrupt estrogen production in pregnancy, affecting pregnancy outcome.

Introduction

Human aromatase (CYP19) converts C19 androgens to aromatic C18 estrogenic steroids (Thompson 1974). In primates, recent findings demonstrate CYP19 activity is critical for early and mid pregnancy maintenance and in regulating parturition in late pregnancy (Albrect 2000; Nathanielsz 1998). Past studies utilized placental microsome tritiated water release assay to assess drug-hormone interactions with estrogen synthesis. Using a high throughput (HT) fluorometric method to detect aromatase inhibitors (Stresser 2000), a panel of azole antifungal agents were tested that are often administered to women of childbearing potential. Findings demonstrate rapid screening potential for chemicals that affect pregnancy outcome as a result of CYP19 inhibition.

Results and Discussion

Assay Validation – Comparisons with the Placental Microsomes

Table 3 lists mean IC_{50} values and compares the HT values with those reported in the literature for placental aromatase tritiated water assay. Rank ordering is identical however, HT assay values are lower for potent inhibitors. Metabolic inhibitor depletion in placental microsomes might explain differences in potency between the two methods (Hakkola, 1996). Binding of inhibitors to reaction vessels walls may occur with very dilute solutions and could underestimate potency. We minimized this possibility by including insect cell control protein (expected to have a solubilizing effect) in all serial dilutions of inhibitor.

IC_{50} Value and Clinical Correlates

Table 3 lists mean IC_{50} values and compares the HT values with the levels achieved in humans following therapeutic dosage. For compounds econazole, bifonazole, sulconazole, clotrimazole, miconazole, ketoconazole and fluconazole the *in vivo* concentrations are within the range of the HT IC_{50} values. Rodents do not express aromatase activity in their placentas during pregnancy [Simpson 1994], so they may not predict toxicity occurring in primate pregnancy. But preclinical reproductive toxicity rodent studies of many antifungal azoles do show delayed labor and embryo- and fetotoxicity (PDR). FDA's Rosa et al (1987), studied pregnancy outcome data from the very large Michigan Medicaid Prescription database after first-trimester exposure to vaginitis drug therapies. Using three separate analyses, miconazole exposure consistently showed a relative risk for spontaneous abortion of 1.4 (95% CI 1.2-1.5) that was independent of the drug therapy indication. This increased relative risk was also noted with the antifungal clotrimazole, but not the polyene antifungal nystatin. Prolongation of gestation was seen in Hungarian epidemiological studies investigating human use of clotrimazole, as an increase in mean gestational age in the drug-exposed group (Czeizel 1999).

Materials and Methods

Incubations with cDNA-expressed enzymes

Assays were conducted in 96-well microplates. Aromatase enzyme (baculovirus/insect cell-expressed) and the substrate DBF was obtained from BD Biosciences Discovery Labware (Woburn, MA). Bifonazole, clotrimazole, econazole, ronidazole, tinidazole, thiabendazole, sulconazole and 1-benzylimidazole were obtained from Sigma-Aldrich. Sulfaphenazole, fluconazole, tetraconazole and itraconazole were obtained from Ultrafine (Woburn, MA), ICN Biomedicals (Aurora, OH), Crescent Chemicals (Islandia, NY) and Research Diagnostics Inc (Flanders, NJ), respectively. A 50% inhibitory concentration (IC_{50}) was determined utilizing 12 wells in each test. A cofactor/serial dilution (C/SD) buffer contained an NADPH-regenerating system and 0.1 mg/mL microsomal protein prepared from insect cells infected with wildtype virus ("control protein"). To the first well in each row, 150 µl of the test compound [dissolved in acetonitrile, or, DMSO (itraconazole only)] and C/SD buffer were added. Compound was diluted serially 1:3 through the 8th well. Wells 9 and 10 contained no inhibitor and wells 11 and 12 were used as controls for background fluorescence [enzyme/substrate (E/S) added after the reaction was terminated]. Plate was then warmed to 37ºC and reaction initiated by the addition of E/S. The E/S mix contained buffer, P450 enzyme (2 pmol/mL final), control protein (0.25 mg/mL final), substrate (0.2 µM final). Reactions were terminated after 30 min by addition of 75 µl 2 N NaOH. Fluorescence signal was measured (ex = 485, em = 538) using a FLUOstar model 403 fluorescence plate reader (BMG LabTechnologies, Inc., Durham, NC). The IC_{50} values were calculated by linear interpolation.

1 Kinetic properties of baculovirus/insect cell-expressed Aromatase BD Supersomes™) and assay parameters for inhibition analysis with Dibenzylfluorescein (DBF)[a]

Substrate	Testosterone	DBF
Metabolite	Estradiol	Flourescein
Apparent K_m (µM)	0.043	0.188
Apparent V_{max} (min^{-1})	8.4	0.32
Substrate concentration for IC_{50} (µM)	NA	0.20
Pmol enzyme per well (200 µl vol.)	NA	0.4
Incubation Time (min)	NA	30

NA – Not applicable
[a] – Data from Stresser et al (2000).

Km values for natural substrate is within 5-fold of fluorometric probe

2 Summary of the IC_{50} values for selected Azole test compounds

	IC_{50} Value (µM)			
Test Compound	Day 1	Day 2	Mean	Range/Mean
Econazole	0.004	0.004	0.004	0.07
Bifonazole	0.008	0.006	0.007	0.14
Sulconazole	0.017	0.014	0.015	0.10
Clotrimazole	0.019	0.017	0.018	0.07
Miconazole	0.034	0.037	0.036	0.04
Tetraconazole	0.22	0.23	0.22	0.02
1-Benzylimidazole	0.59	0.53	0.56	0.06
Ketoconazole	1.1	2.9	2.0	0.44
Fluconazole	28.4	25.2	26.8	0.06
Sulfaphenazole	115	144	129	0.11
Thiabendazole[b]	191	199	195	0.02
Tinidazole[c]	923	726	824	0.12
Itraconazole	> 100	> 100	> 100	-
Metronidazole	> 100	> 100	> 100	-
Ronidazole	> 1000	> 1000	> 1000	-
			mean	0.10

[a] IC_{50} values were determined in duplicate on separate days
[b] One reliplicate IC_{50} value from each day was near, but greater than 200 µM; values calculated on assumption that IC_{50} value for that replicate was 200 µM.
[c] One reliplicate IC_{50} value was near, but greater than 1000 µM; Mean values were calculated on assumption that IC_{50} value was = 1000 µM.

3 Mean IC_{50} values of several azoles sorted by rank order.
Placental microsomal tritiated water assay IC_{50} values from the literature are listed for comparison.

		IC_{50} Value (µM)	IC_{50} Value (µM)	human (µM)
Test Compound	Application	HT Mean	Placenta Mic	serum/tissue
Econazole	antifungal	0.004	0.24[1]	1% absorb[4]
Bifonazole	antifungal	0.007	0.29[1]	0.05 serum[5] / 0.65 skin[6]
Sulconazole	antifungal	0.015		14% absorb[7]
Clotrimazole	antifungal	0.018	0.39[1]	0.03 serum / >100 vag[4]
Miconazole	antifungal	0.036	0.46[1], 0.06 (Ki)[2]	0.024 serum / 1.4% vag[4]
Tetraconazole	fungicide	0.22		
1-Benzyl imidazole	synthetic intermediate	0.561		
Ketoconazole	antifungal	2	6.6[1]	6 -12 serum
Fluconazole	antifungal	26.8	60[3]	20 serum / uterus[8]
Sulfaphenazole	antimicrobial	129	> 100	
Thiabendazole	antihelmintic	195	> 100	
Tinidazole	antiprotozoal	824	> 100	> 100
Metronidazole	antiprotozoal	> 100	> 100	
Itraconazole	antifungal	> 1000		
Ronidazole	antiprotozoal	> 1000		

[1] average of values for two androgen substrates (Ayub, 1998)
[2] Mason, 1985
[3] Kragie, 1996
[4] PDR
[5] Lackner, 1989
[6] Patzschke, 1983
[7] Fromtling, 1988
[8] Mikamo, 1999

Summary and Conclusions

1. Aromatase activity is critical for successful primate pregnancy.
2. Azole drugs given to pregnant women, inhibit aromatase. Plasma and tissue levels of some azole drugs following oral or topical administration are at or above these IC_{50} values. These include the oral agents **fluconazole** and **ketoconazole**, and the topical agents **econazole**, **bifonazole**, **clotrimazole**, **miconazole**, and **sulconazole**.
3. Therefore, some azole drugs may disrupt estrogen production and affect pregnancy outcome. Clinical and epidemiology data support these *in vitro* toxicology results.
4. Using recombinant CYP19 and the fluorometric substrate DBF, our findings demonstrate rapid screening potential for chemicals that may affect pregnancy outcome as a result of CYP19 inhibition.

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