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THE USE OF RAT LEYDIG TUMOR (R2C) AND HUMAN HEPATOMA (HEPG2) CELLS TO EVALUATE POTENTIAL INHIBITORS OF RAT AND HUMAN STEROID AROMATASE

KATHLEEN M. DOODY[a], BARBARA A. MURRY[a] and J. IAN MASON*[a,b]

Cecil H. and Ida Green Center for Reproductive Biology Sciences, [a] Department of Obstetrics and Gynecology and [b] Department of Biochemistry, The University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, Texas 75235

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The efficacies of 10-propargylestr-4-ene-3,17-dione (PED), 4-hydroxyandrostenedione (4-OHA) and the imidazole broad spectrum antimycotic drugs, econazole, imazalil, miconazole and ketoconazole, to inhibit the steroid aromatase activities of rat Leydig tumor (R2C) cells and human hepatoma (HEPG2) cells have been determined. The analysis of inhibition of steroid aromatase activity of intact cells provided further insight into the potential use of such drugs to block cellular estrogen synthesis. The IC_{50} values for the inhibition of aromatase activity of R2C cells by econazole, imazalil, miconazole, ketoconazole, 4-OHA and PED were 4, 9, 40, 1100, 11 and 10 nM, respectively. These drugs also inhibited the steroid aromatase activity of HEPG2 cells with corresponding IC_{50} values of 13, 27, 20, 15000, 2 and 2 nM, respectively; these findings were suggestive that the steroid aromatase of rat has many similarities to the human enzyme in its interaction with putative inhibitory compounds. Importantly, however, ketoconazole inhibited the rat aromatase more effectively than it did the human enzyme, while PED and 4-OHA were less effective inhibitors of the rat enzyme compared to that of human. These findings indicate differences in the potencies of various drugs to inhibit estrogen biosynthesis in human and rat cells. These may relate to differences in the two aromatase systems and/or differences in the stability of the drugs in the human hepatoma and rat Leydig tumor cells.

KEY WORDS: Aromatase, aromatase inhibitors, rat Leydig tumor, human hepatoma, R2C, HEPG2.

INTRODUCTION

Econazole, imazalil, miconazole, and ketoconazole are imidazole antimycotic agents effective against a wide range of fungal pathogens. These drugs effectively inhibit ergosterol biosynthesis in yeast cells by inhibiting the sterol 14α-demethylase, a microsomal cytochrome P-450-dependent system.[1] This inhibition causes an accumulation of 14α-methylsterols which incorporate into the bilayer of fungal membranes. The accumulation of 14α-methylsterol is believed to disrupt the close packing of acyl chains of phospholipids resulting in limited fungal growth.[2] Imidazole antimycotic agents have been shown to inhibit a number of cytochrome P-450-dependent steroidogenic enzyme activities in gonadal, hepatic, adrenal and placental tissues.[3–8]

* Correspondence.

The aim of the present research was to evaluate the effectiveness of putative aromatase inhibitors to inhibit the steroid aromatase activities of human hepatoma cells and rat Leydig tumor cells. We had previously found that the aromatase of extra-placental human tissue, *viz* fetal liver, appeared very similar to human placental aromatase.[9]

MATERIALS AND METHODS

Chemicals

Econazole (1-[2-([4-chlorophenyl]methoxy)-2-(2,4-dichlorophenyl)ethyl]-1H-imidazole) nitrate and miconazole (1-[2,4-dichloro-β-([2,4-dichlorobenzyl]oxy)-phenethyl]imidazole) nitrate were obtained from the Sigma Chemical Company (St. Louis, MO). Ketoconazole ([$\pm$]-*cis*-1-acetyl-4-[4-([2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)1,3-dioxolan-4-yl)-methoxy)phenyl]piperazine) and imazalil (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1H-imidazole) were supplied by Janssen Life Products Inc. (Piscataway, NJ). Dr Angela Brodie (University of Maryland, Baltimore, MD) kindly supplied a sample of 4-hydroxyandrostenedione (4-androstan-4-ol-3,20-dione). Dr Douglas Covey (University of Washington School of Medicine, St. Louis, MO) generously supplied 10-propargylestr-4-ene-3,17-dione. [1β-^{3}H]Androst-4-ene-3,17-dione (22 Ci/mmol) was supplied by NEN Dupont (Boston, MA). All other chemicals were of reagent grade quality purchased from scientific supply houses.

Cells

The rat Leydig testicular tumor (R2C) cells and the human hepatoblastoma (HEPG2) cells were obtained from the American Type Tissue Culture Collection (Rockville, MD). The cells were routinely cultured in Dulbecco's Modified Eagle's (DME) medium (4.5 g/l glucose) that contained calf bovine serum (5%). Protein content was measured by the Bradford method using bovine serum albumin as the standard.[10]

Steroid Aromatase Activity

Confluent cell monolayers of R2C or HEPG2 cells in DME medium supplemented with ITS$^+$ (Collaborative Research, Waltham, MA) were incubated with [1β-^{3}H]androstenedione (0.5 μCi, 150 pmoles) in a final volume of 1.0 ml. The reaction was initiated by the addition of androstenedione and proceeded for 2 h at 37°C. The assays were terminated by removal of a 0.9 ml aliquot of medium followed by the addition of trichloroacetic acid (0.9 ml, 30% w/v) to denature the protein. After the addition of chloroform (5 ml) and centrifugation, aliquots (0.9 ml) of the aqueous phase were mixed with an equal volume of an activated charcoal suspension (5% w/v, Norit A). After centrifugation at 700 $\times$ *g* for 15 min, aliquots (0.9 ml) of the supernatant were transferred to scintillation vials and the radioactivity quantified by liquid scintillation spectrometry. This assay method is based on the specific loss of tritium from the 1β position of tritiated androstenedione to form ^3H_2O during the process of aromatization.[11] Insignificant incorporation of label into the water phase, and, thus, formation of tritiated water from [1β-^{3}H]androstenedione in homogenates of placenta or liver was apparently associated strictly with aromatization.[9]

RESULTS

Time course of aromatization of androstenedione in R2C and HEPG2 cells

The rates of release of ^3H_2O from [1-^{3}H]androstenedione by R2C and HEPG2 cells, as a function of time, are presented in Figure 1. The rates for both cell types appeared

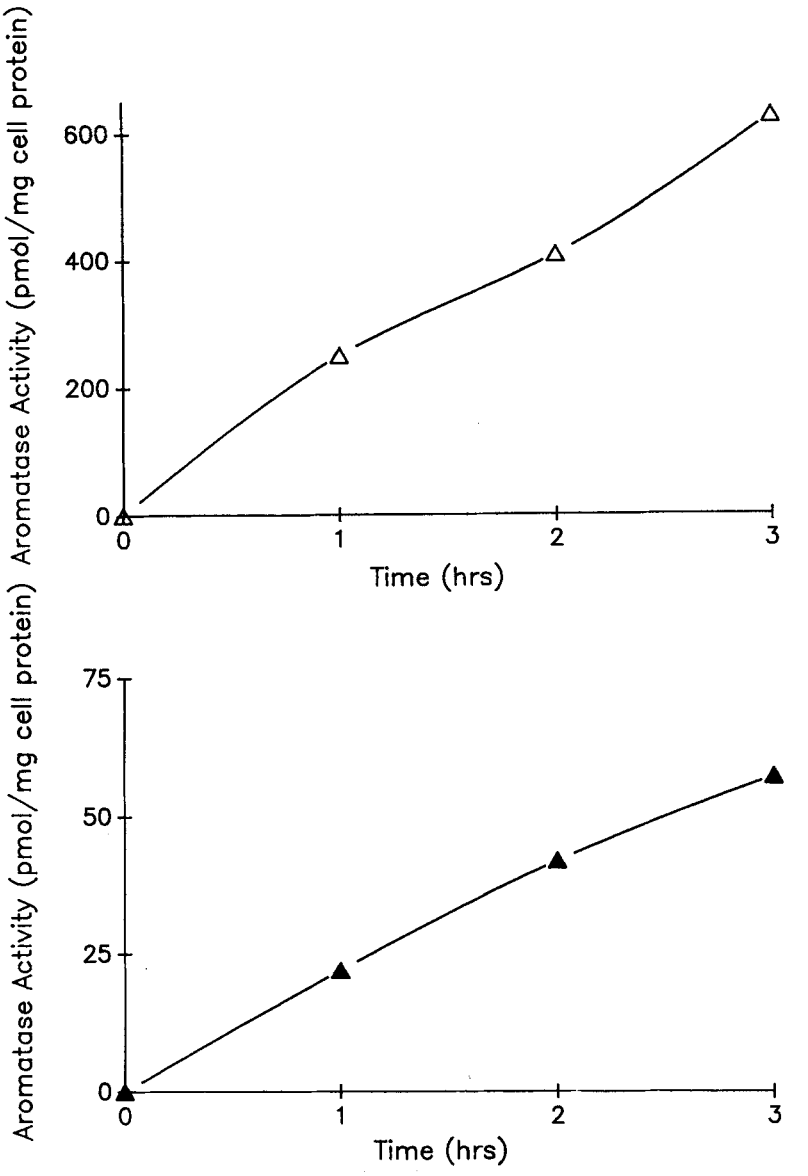

FIGURE 1 Time course for the release of ^3H_2O when R2C (A) or HEPG2 (B) were incubated in DME medium that contained [1β-^{3}H]-androstenedione (0.15 μM). Triplicate samples were obtained at each time point.

essentially linear over the 3 h assay period. Thus, in subsequent assays, a 2 h assay period was routinely used.

Inhibition of aromatase activity of R2C cells by imidazole and steroidal drugs

To determine the relative potency of the imidazole antimycotics, as well as PED and 4-hydroxyandrostenedione, to inhibit the steroid aromatase activity of R2C cells, we determined the aromatase activity of the cells over a 2 h period in the presence of increasing amounts of these compounds. In Figure 2, a typical plot to determine IC_{50} values, the amount of inhibitor required to produce 50% inhibition of aromatase activity, is represented. Econazole, 4-hydroxyandrostenedione, PED and imazalil were found to be very potent inhibitors of aromatase activity, miconazole was a less effective inhibitor, while ketoconazole was a weaker inhibitor of the enzyme.

Similar titrations of the aromatase activity of HEPG2 cells with various doses of the six effectors were performed and IC_{50} values established. In Table 1, the IC_{50} values for the amount of effector required to produce 50% inhibition of aromatase activities of HEPFG2 and R2C cells are presented. PED and 4-hydroxyandrostenedione were the most effective inhibitors, econazole, imazalil and miconazole somewhat less effective, while ketoconazole was a relatively ineffective aromatase inhibitor.

DISCUSSION

Sato and colleagues reported that the rat Leydig R2C tumor cell line synthesized estrogen on the addition of androstenedione, a finding indicative that these cells expressed the steroid aromatase system.[12] We confirmed that observation and herein report that R2C cells (Figure 1A) express levels of aromatase activity (200 $pmol.h^{-1}.mg^{-1}$ cell protein) that are comparable to those determined in human choriocarcinoma cell lines. Recognized selective and potent inhibitors of the human aromatase, *viz* PED,[13] 4-hydroxyandrostenedione,[14] econazole[9] and imazalil,[9] effectively blocked the release of ^3H_2O from [1β-^{3}H]-androstenedione (Figure 2); a finding indicative that we were indeed assaying aromatase activity. Econazole and imazalil suppressed this rat aromatase activity more effectively than 4-hydroxyandrostenedione or 10-propargylestr-4-ene-3,17-dione.

We have reported earlier that aromatase activity is present not only in human placental microsomes but also in human fetal liver microsomes. Because the human hepatoma cell line, HEPG2, is derived from a hepatoblastoma of presumed fetal

TABLE 1
IC_{50} values for aromatase inhibitors in rat Leydig tumor (R2C) and human hepatoma (HEPG2) cells

	R2C	HEPG2
	IC_{50} (μM)	
Econazole	0.004	0.013
Imazalil	0.009	0.027
Miconazole	0.040	0.02
Ketoconazole	1.1	15
PED	0.011	0.002
4-OHA	0.01	0.002

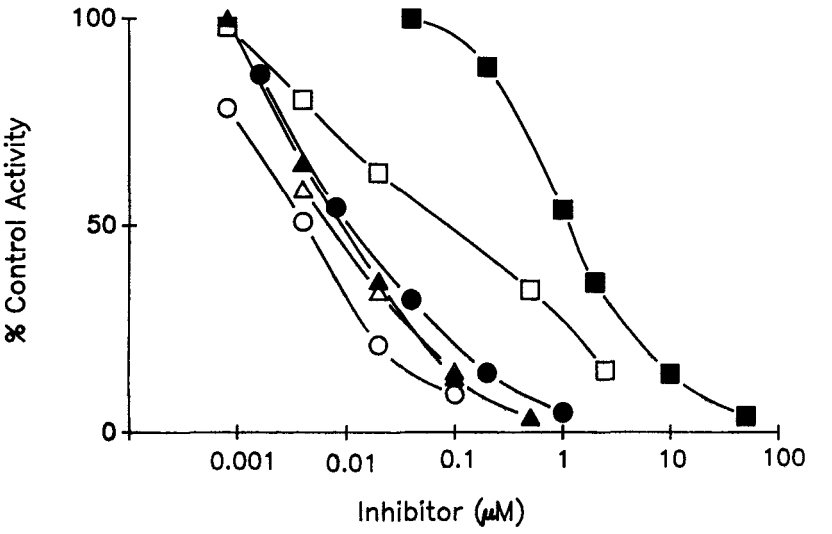

FIGURE 2 Dose-response curves for the inhibition of aromatase activity of R2C cells by econazole (○), 4-hydroxyandrostenedione (△), PED (●), imazalil (▲), miconazole (□) and ketoconazole (■). The concentration of androstenedione was 0.15 μM. The control activity of the R2C cells was 400 pmol.h^{-1}.mg^{-1} cell protein.

origin, we evaluated the level of aromatase activity present in this cell line. We found that the HEPG2 cells catalyzed the release of ^3H_2O from [1β-^{3}H]-androstenedione (Figure 1B) and, importantly, this release of ^3H_2O was inhibited by known potent inhibitors of human placental aromatase. Under the assay conditions employed, econazole, imazalil, miconazole, PED and 4-hydroxyandrostenedione were found to be very effective inhibitors of HEPG2 aromatase activity (Table I).We previously have shown that the site of the inhibitory activity of miconazole appears to be the cytochrome P-450 component of the aromatase monooxygenase complex. The type II spectral perturbation observed upon the addition of miconazole to placental microsomal cytochrome P-450 is strongly suggestive of the formation of a nitrogenous ligand to the P-450, most probably the interaction of an imidazole nitrogen of miconazole with the hemoprotein. The binding of miconazole to human placental steroid aromatase appears reversible and competitive with substrate.[7] We anticipate a similar mechanism of inhibition of HEPG2 aromatase by the imidazole drugs.

We reported previously that imidazole antimycotic agents demonstrate intriguing selective abilities to inhibit the various hydroxylases involved in the metabolism of androstenedione in rat liver.[8] We report here that ketoconazole is a more potent inhibitor of the aromatase activity of rat Leydig tumor cells than of human HEPG2 tumor cells. Similarly econazole and imazalil appear to inhibit the rat enzyme more effectively than they suppress the human enzyme. In contrast, 4-hydroxyandrostenedione and PED inhibit the aromatase activity of the rat tumor cells less effectively than they suppress the activity of human tumor cells. The relative differences of these effectors to alter aromatase activity in these two tumor cell systems may relate, in part, to the rate of metabolism or disposition of these compounds in the cells. The disposition of potential drugs, however, is a most important determinant of the efficacy of a drug. Because many potential aromatase inhibitors are screened initially

in rats, we believe the assessment of inhibitory potency of a compound in the rat Leydig R2C cell line and a human tumor cell line, such as HEPG2, that both express aromatase, will facilitate the development of useful aromatase inhibitors. The chemical features resulting in the specificity of imidazole antimycotic compounds to inhibit different cytochrome P-450s are not yet clear. A comparison of chemical structures of these drugs affords no apparent simple structure-activity relationship. We believe the results of our study are suggestive that imidazole drugs such as econazole and imazalil are comparable with 4-hydroxyandrostenedione and 10-propargylestr-4-ene-3,17-dione as inhibitors of estrogen biosynthesis in situations that require suppression of estrogen formation. Other modifications of these drugs may result in compounds that may prove to be more selective and potent inhibitors of cytochrome P-450-dependent steroid hydroxylase enzymes. The investigative use of cell lines such as R2C and HEPG2 may facilitate those developments.

Acknowledgments

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