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ENZYME INACTIVATION BY POTENTIAL METABOLITES OF AN AROMATASE-ACTIVATED INHIBITOR (MDL 18,962)

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MDL 18,962, 19-acetylenic androstenedione, is an enzyme-activated inhibitor of estrogen biosynthesis which is in Phase I clinical evaluations as a potential therapeutic agent for estrogen-dependent cancers. 19-Acetylenic analogs corresponding to the major metbolites of androstenedione were synthesized as potential metabolites of MDL 18,962. These compounds were 19-acetylenic testosterone, the product of 17β-hydroxy steroid oxidoreductase, 6β-hydroxy- and 6-oxo-19-acetylenic androstenedione, products of P_{450} steroid 6β-hydroxylase and alcohol dehydrogenase, respectively. All of these analogs showed time-dependent inactivation of human placental aromatase activity. The time-dependent K_i and $t_{1/2}$ at infinite inhibitor concentration (τ_{50}) were 4.3 nM, 12.0 min for MDL 18,962; 28 nM, 7.8 min for 17-hydroxy analog; 13 nM, 37 min for 6β-hydroxy analog; and 167 nM, 6.1 min for the 6-oxo analog. The 19-acetylenic testosterone, a confirmed metabolite from primate studies, was 25% as efficient as MDL 18,962 for aromatase inactivation, while 6β-hydroxy- and 6-oxo analogs were 11% and 5%, respectively as efficient as their parent compound. These data indicate that first-pass metabolism of MDL 18,962 does not cause an obligatory loss of time-dependent inhibition of human aromatase activity.

KEY WORDS: Aromatase Inhibition, MDL 18,962, Estrogen Biosynthesis, P_{450} Hydroxylation, steroid 17β-Dehydrogenase

INTRODUCTION

MDL 18,962 has been shown to be an enzyme inhibitor of estrogen biosynthesis in rodent and primate models.[1-3] Human hepatic P_{450} monooxygenases metabolize androgens mainly via 6β-hydroxylation with limited formation of 7α- and 16α-hydroxylated metabolites.[4,5] Therefore, three potential metabolites of MDL 18,962, 19-acetylenic testosterone, 6β-hydroxy, and 6-oxo-19-acetylenic androstenedione, were synthesized and evaluated for their competitive and time-dependent inhibition of human placental aromatase.

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Abbreviations: **MDL 18,962**, 19-acetylenic androstenedione, 10-(2-propynyl)estr-4-ene-3,17-dione, Chemical Abstract Registry No. 77016-85-4; **19-AT**, 19-acetylenic testosterone, 17β-hydroxy-10-(2-propynyl)estr-4-en-3-one; **6-OH-19-AA**, 6-hydroxy-19-acetylenic androstenedione, β-hydroxy-10-(2-propynyl)estr-4-ene-3,17-dione; **6-oxo-19-AA**, 6-oxo-19-acetylenic androstenedione, 6-oxo-10-(2-pro-pynyl)estr-4-ene-3,17-dione.

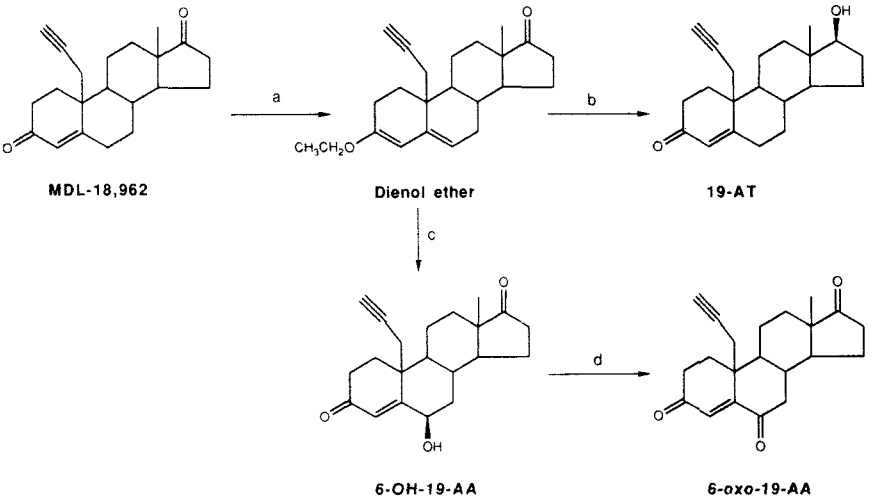
MATERIALS AND METHODS

Chemicals and Reagents

All chemical reagents for aromatase assays were as previously described.[1,6] Labeled substrate [1-^{3}H]-androstenedione (28.2 Ci/mmol) was obtained from NEN Research Products (Boston, MA), and its radiochemical purity (99+%) confirmed by an HPLC system[7] prior to use. Steroidal aromatase inhibitors were synthesized and characterized by standard analytical methods at Merrell Dow Research Institute (Scheme 1). MDL 18,962 was prepared as published[8] and was converted to the dienol ether analog by the method of Burn *et al.*[9] Sodium borohydride reduction with subsequent hydrolysis of the enol ether afforded 19-AT. Exposure of a stirred ethanolic solution of dienol ether of MDL 18,962 to direct sunlight[10] provided the 6-hydroxy deriative, 6-OH-19-AA, which was oxidized to the ketone, 6-oxo-19-AA, by the Swern procedure.[11]

19-Acetylenic testosterone (19-AT): m.p. 118-120°C; IR (KBr) ν 3420, 3330, 2110, 1665 cm^{-1};' NMR ($CDCl_3$) δ 0.83 (s, 3 H, 18-CH_3), 2.01 (t, 1 H, J = 2 Hz, H-C≡C), 3.65 (br t, 1 H, J = 7 Hz, H_{17}), 5.89 (s, 1 H, H_4), UV (EtOH) λ_{max} 241 nm (ε = 15,500). Anal. Calcd. for $C_{21}H_{28}O_2$: C, 80.73; H, 9.03. Found: C, 80.77; H, 9.23%.

6β-OH-19-Acetylenic androstenedione (6-OH-19-AA): m.p. 204–206°C, IR (KBr) ν 3495, 3250, 2120, 1730, 1680 cm^{-1}; NMR ($CDCl_3$) δ 0.92 (s, 3 H, 18-CH_3), 2.12 (t, 1 H, J = 2.5 Hz, HC≡C), 4.40 (br s, 1 H, H_6), 6.00 (s, 1 H, H_4); MS (70 ev) *m/e* 326 (M^+), 269 (100%); MS (CI/CH_4) *m/e* 327 (M + H, 100%); UV (EtOH) λ_{max} 237 nm (ε = 11,700). Anal. Calcd for $C_{21}H_{26}O_3$: C, 77.27; H, 8.03. Found: C, 77.18; H, 8.06%



SCHEME 1. Chemical Synthesis of Inhibitors.

Reagents and conditions for each reaction were: (a) $(EtO)_3CH$/pTsOH/EtOH/THF, 25°C, 1.25 h; (b) $NaBH_4$, EtOH/THF, 10% aq. NaOH, 25°C, 2 h; (c) EtOH, direct sunlight, 6 h; (d) (i) ClCOCOCl, DMSO, CH_2Cl_2, −50°C; (ii) Et_3N.

6-Oxo-19-acetylenic androstenedione (6-oxo-19-AA): m.p. 176-178°C; IR (KBr) ν 3300, 2120, 1735, 1690, 1673 cm^{-1}; NMR ($CDCl_3$) δ 0.98 (s, 3 H, 18-CH_3), 2.12 (t, 1 H, J = 2 H, HC≡C), 6.39 (s, 1 H, H_4); MS (70 ev) *m/e* 324 (M^+), 282 (100%); MS (CI/CH_4) *m/e* 325 ($M^+ + 1$, 100%); UV (EtOH) λ_{max} 247 nm (ε = 8620). Anal. Calcd for $C_{21}H_{24}O_3$: C, 77.75; H, 7.46. Found: C, 77.79; H, 7.51%.

Enzyme Assays

Lyophilized microsomes (39% protein) with an enzyme activity of 34.6 pmoles estrogen/min/mg protein were prepared from term human placentas.[1] Time-dependent aromatase assays were prepared as previously described[1,6] using 500 μM androstenedione as substrate. Analogs of MDL 18,962 were evaluated at 5–100 nM. MDL 18,962 at 5 nM was evaluated in these assays as a concurrent positive reference. Kinetic data from previous experiments[2] with MDL 18,962 were utilized for comparative purposes. The isotopic distribution for [1-^{3}H]-androstenedione was estimated to be 57% 1β from the percentage of ^3H_2O formed during a 90 min aromatase assay. This distribution was confirmed by product isolation of [^{3}H]-estrogens by HPLC.[7] Data expressed as moles of estrogen formed were adjusted for isotopic distribution. Duplicate assays were conducted for each inhibitor concentration. The amount of ^3H_2O product measured was corrected for assay background using assay tubes with solvent-inactivated enzyme. Time-dependent enzyme kinetic data were expressed as percent of control enzyme activity which adjusts for inherent loss of aromatase activity (16%) during the 20-min preincubation phase.

Estimates of enzyme affinities for pseudo-substrate analogs were determined from 0 min preincubation data as previously described[6] using substrate concentration of 500 nM and K_m for androstenedione of 40 nM. Time-dependent enzyme inhibition constants for apparent $K_{i(inact)}$ were estimated via the method of Kitz and Wilson[12] from time-dependent inhibition experiments. Least-squares regression analysis of the reciprocal of inhibitor concentration versus the respective enzyme half-life of enzyme inactivation, $t_{1/2}$, at each inhibitor concentration was utilized for these $K_{i(inact)}$ estimates.[13] Statistical analyses[14,15] were applied to the time-dependent inhibition rates to determine significance of slope and degree of linearity.

RESULTS

The 6β-hydroxy and 17β-hydroxy analogs had apparent affinity values for aromatase which were equivalent to or lower than the apparent K_m for androstenedione, 40 nM. The 6-oxo- analog had approximately 10-fold weaker affinity for the enzyme than the substrate. In inactivation studies all compounds produced time-dependent, pseudo-first-order inactivation of aromatase (Figure 1). Kinetic analysis of this inactivation process[12,13] provided apparent $K_{i(inact)}$ which represents the inhibitor concentration required to produce half-maximal rate of inactivation. The first-order rate constant for inactivation (k_{cat}) and the half-time of inactivation (τ_{50}) at infinite inhibitor concentration are shown in Table 1. The efficiencies of these analogs as inactivators of aromatase were compared to MDL 18,962 using the ratio of $k_{cat}/K_{i(inact)}$. The 17β-hydroxy analog was approximately one-fourth as efficient as MDL 18,962, while the 6β-hydroxy was one-tenth and the 6-oxo was about one-half of the 6β-hydroxy analog.

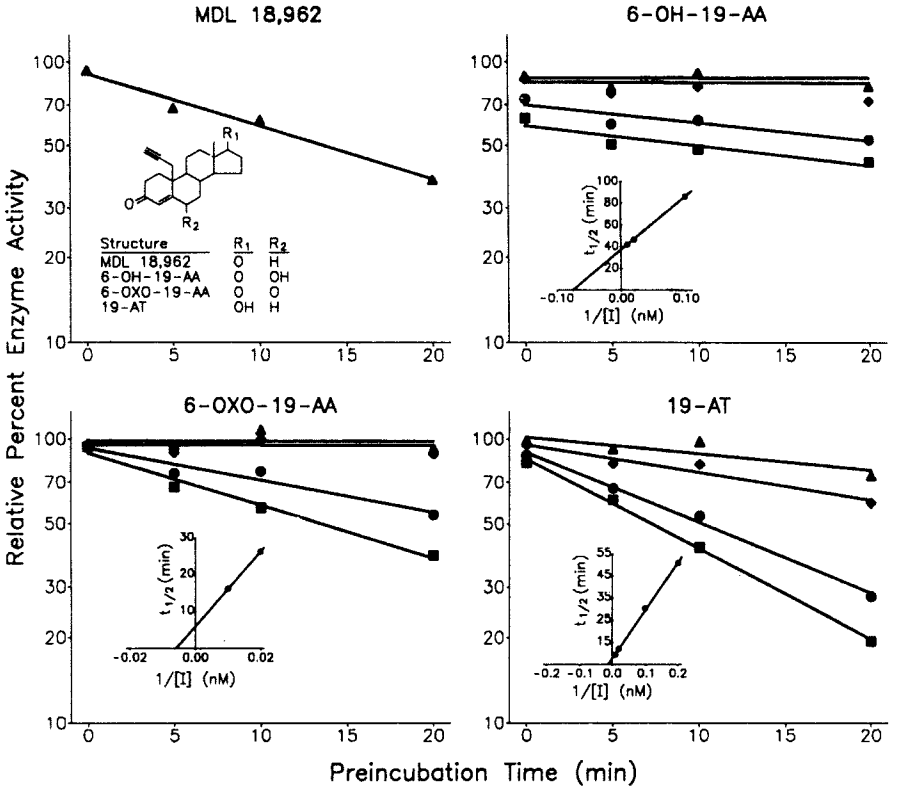


FIGURE 1 Time-dependent inhibition of aromatase. Steroidal inhibitors were preincubated with 136 μg of human placental microsomal protein and NADPH-generating systems for 0–20 min prior to addition of [1-^{3}H]-androstenedione (500 nM; 0.8 μCi) for 10 min assay at 25°C. Product formed was quantitated by ^{3}H release assay. The inserts are Kitz-Wilson plots[12] of 1/[I] vs. $t_{1/2}$ for 5 nM▲; 10 nM◆; 50 nM●; 100 nM■.

TABLE I
Kinetic Analysis of MDL 18,962 Analogs for Inhibition of Placental Aromatase Inhibition.

Inhibitors	$K_{i(inact)}$[1] (nm)	τ_{50} (min)	k_{cat} (sec^{-1})	$k_{cat}/K_{i(inact)}$	Ranking[2] (%)
MDL 18,962	4.3	12.0	9.6×10^{-4}	223,000	100
6-OH-19-AA	13.4	36.7	3.1×10^{-4}	24,000	11
6-OXO-19-AA	167.1	6.1	1.9×10^{-3}	11,000	5
19-AT	28.1	7.8	1.5×10^{-3}	53,000	24

[1] Estimated from pseudo-first-order kinetics using Kitz-Wilson plots.[12]
[2] Compounds compared to MDL 18,962 = 1.0. Time-dependent data[2] for MDL 18,962 was used to rank the relative efficiency of aromatase inactivation.

DISCUSSION

Lowering of estrogen levels *in vivo* via enzyme inhibitors of aromatase has been shown to be an effective pharmacological strategy in treatment of clinical conditions associated with elevated estrogen biosynthesis.[16,17] MDL 18,962 has been characterized as an enzyme-activated inhibitor of aromatase[1,2] and shown to be active *in vivo*[2,3] with long-lasting biochemical and pharmacological effects.[18] When female baboons were intravenously injected with [^{14}C]-MDL 18,962, the inhibitor was rapidly converted (97%) via first-pass metabolism to multiple radioactive urinary components. These metabolites were separated by HPLC chromatography and analyzed by mass spectrometry. The two major metabolites of [^{14}C]-MDL 18,962 representing approximately 30% each of the total [^{14}C] were a steroidal ring hydroxylated compound and the 17β-hydroxy analog (unpublished data).

The conversion of 19-acetylenic androstenedione (MDL 18,962) to 19-acetylenic testosterone would occur via the reversible 17β-hydroxy steroid oxidoreductase. Steroidal B-ring hydroxy metabolites would predictably be the 6α-hydroxy, 6β-hydroxy, or 7α-hydroxy analog formed via hepatic metabolism. The 6β-hydroxylation is the major hydroxylation pathway in human liver microsomes.[19] In contrast to the more intensely studied rat hepatic cytochrome P_{450} systems,[4,20–22] no sex-related differences were observed in metabolism of testosterone at the C6 position in human liver.[5] The 6-oxo analog could presumably be formed from the 6β-hydroxy compound via nonspecific hepatic alcohol dehydrogenase.[23]

Therefore the 6β-hydroxy, 6-oxo, and the 17β-hydroxy analogs of MDL 18,962 were synthesized and evaluated for aromatase inhibitory activity. These potential metabolites of MDL 18,962 exhibited time-dependent and concentration dependent inhibition of aromatase activity of human placental microsomes. The ratio of the rate of enzyme inactivation (k_{cat}) to half-maximal inhibitor concentration $K_{i(inact)}$ permits a rank ordering of these inhibitors in relationship to MDL 18,962, thus MDL 18,962 $\gg$ 17β-hydroxy > 6β-hydroxy > 6-oxo analogs in relative inhibitory activity. These data suggested that biotransformation of MDL 18,962 does not cause an obligatory loss of its enzyme-activated inhibiton of aromatase.

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