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METABOLISM OF CLOMIPHENE IN THE RAT

ESTROGEN RECEPTOR AFFINITY AND ANTIESTROGENIC ACTIVITY OF CLOMIPHENE METABOLITES*

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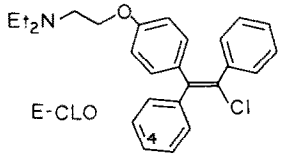
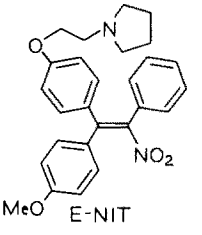
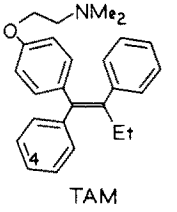
Abstract—Incubation of the nonsteroidal antiestrogen clomiphene with rat liver microsomes resulted in the formation of the 4-hydroxy-, *N*-desethyl-, and *N*-oxide metabolites, in qualitative contrast to results previously obtained analogously with rabbit microsomes, with which only the first two metabolites were detected. Metabolites were characterized by thin-layer chromatography in comparison with synthetic standards. They were similarly compared using low resolution electron ionization mass spectrometry, except for the *N*-oxide which was best characterized by fast atom bombardment mass spectrometry. Oral administration of clomiphene resulted in no detectable urinary elimination of the drug or its metabolites; 4-hydroxyclomiphene was the sole detectable elimination product in fecal extracts. The relative uterine cytosol estrogen receptor binding affinities, at 4°, of 4-hydroxyclomiphene and the E-isomers of clomiphene, desethylclomiphene, and clomiphene *N*-oxide were, in turn, 331, 0.71, 0.62, and 0.88 (estradiol = 100). In the 3-day immature rat uterotropic assay, 4-hydroxyclomiphene had no significant uterotropic effect at doses up to 50 μg/day, but substantially inhibited that of estradiol (0.5 μg/day) at doses of 2 μg/day.

Clomiphene (CLO), a mixture of E-2-[*p*-(2-chloro-1,2-diphenylvinyl)phenoxy]triethylamine (E-CLO)‡ and its geometric isomer, is currently used clinically to stimulate fertility in anovulatory women [1, 2]. Although its mechanism of action is unknown, it has been shown in experimental studies to possess both estrogenic and antiestrogenic properties. For example, it increases the responsiveness of cultured anterior pituitary cells to gonadotropin releasing hormone in a way similar to that of estradiol [3, 4]. In the immature rat, the isomers of CLO stimulate uterine growth, but antagonize partially that produced by estradiol [5]. And, in a clinical study, CLO lowered cytosol endometrial estrogen and progestin receptor concentrations in postmenopausal women on estrogen replacement therapy [6].



The ability of CLO and related triarylethylenes to antagonize the growth-promoting effect of estradiol on target tissue has focused attention on the potential application of these compounds as therapeutic alternatives to surgery in estrogen-dependent cancers [1, 7]. E-CLO was effective in suppressing dimethylbenzanthracene-induced rat mammary tumors [8] and in inhibiting diethylstilbestrol-induced tumorigenesis in Syrian Golden Hamster kidney [9]. In clinical trials, CLO was shown to cause tumor regression in 39% of patients with advanced breast cancer [10]. However, a relatively high incidence of side effects associated with the treatment regimen necessary for suppression of tumor growth limited its potential for this clinical use [11].





The biological activity of several related triarylethylene antiestrogens has been suggested to be mediated in part through metabolites, especially phenolic ones [12, 13]. Thus, in the rat, *O*-demethylation of nitromiphene (NIT) and 4-hydroxylation of tamoxifen (TAM) generate phenolic metabolites with respective estrogen receptor binding affinities 12 and 142 times greater than those of the parent compounds [12, 13]. And, in whole animal studies with immature rats, a diminution in the differences between the antiestrogenic activities of NIT and TAM and their respective phenolic metabolites has been observed, presumably due to *in vivo* conversion to these metabolites [5, 12]. We wanted to determine if CLO was metabolized to an analogous phenolic

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‡ In CLO and other chlorotriarylethylenes, the E-isomers are those in which the benzene ring bearing the basic side chain and the chloro group are *trans* to each other.

metabolite in the rat and, if so, to determine the antiestrogenic activity of this metabolite, along with an estimation of its uterine cytosol estrogen receptor binding affinity in comparison with those of E-CLO and other non-phenolic metabolites. Previous studies have shown E-CLO to be converted to 4-hydroxy and *N*-desethyl metabolites in the presence of rabbit liver microsomes [14].

MATERIALS AND METHODS

Chemicals. E-CLO citrate was a gift from Merrell-Dow Laboratories, Cincinnati, OH. Analysis (as the free base) by NMR showed it to be the pure E-isomer. The preparation of the E-isomers of desethyl CLO and CLO *N*-oxide has been reported [14]. Biochemicals and specialty chemicals, including clomiphene citrate, were purchased from the Sigma Chemical Co., St. Louis, MO, and the Aldrich Chemical Co., Milwaukee, WI.

Analytical equipment. Proton nuclear magnetic resonance (NMR) spectra were recorded using a JEOL JNM/MX-90Q(I) high resolution Fourier transform spectrometer with $CDCl_3$ as solvent and tetramethylsilane as internal standard. Fast atom bombardment positive ion mass spectra of synthetic and metabolic CLO *N*-oxides were obtained using a Varian MAT 731 mass spectrometer equipped with an Ion Tech atom gun which produced a beam of neutral xenon atoms of 6–7 kV [15]. The ion source temperature was 35–40°, the accelerating voltage was 8 kV, and the resolution (M/ΔM) of the instrument was 1200. Approximately 1 μl of a solution of the sample in methanol–glycerol (1:1, v/v) was applied to the probe tip.

Chromatography. Analytical and preparative thin-layer chromatography (TLC) was carried out using, in turn, 5 × 20 cm (0.25 mm thickness) and 20 × 20 cm (1 mm thickness) silica gel GF 254 plates supplied by Analtech, Inc., Newark, DE. Unless otherwise indicated, the solvent for TLC was chloroform–methanol–28% aqueous ammonia (95:5:0.5, v/v).

In vitro *metabolism studies.* Microsomes from livers of male Sprague–Dawley rats (200–250 g) were prepared as described [16]. Incubations were carried out in 25-ml Erlenmeyer flasks. The standard incubation mixture contained, per 5 ml, microsomal protein (10 mg), KCl (600 μmoles), potassium phosphate buffer (400 μmoles), and an NADPH-generating system consisting of NADP (2 μmoles), glucose-6-phosphate (40 μmoles), $MgCl_2$ (25 μmoles), and glucose-6-phosphate dehydrogenase (2.6 units). After a 3-min preincubation at 37°, 25 μl of 0.2 M CLO citrate in *N,N*-dimethylacetamide was added to give a final CLO concentration of 1 mM. The mixture was shaken at 70 cycles/min in a metabolic shaker for 30 min. Control incubations did not contain the NADPH-generating system.

Extraction, and chromatographic detection and isolation of metabolites from incubation mixtures were carried out as described previously [16]. Chromatographic and mass spectral characteristics of purified metabolites in comparison with synthetic standards are summarized in Table 1.

In vivo *studies.* A solution of 10 mg of E-CLO citrate in 50 μl of *N,N*-dimethylacetamide was diluted with 0.45 ml of peanut oil, and the resulting opalescent solution was given orally to a male Sprague–Dawley rat (275 g). The rat was kept in a standard metabolism cage and allowed unlimited food and water. Urine and feces were collected daily for 4 days and stored in the freezer. (A control study was carried out similarly in which the animal received vehicle only.) A 5-ml aliquot of the pooled urine was adjusted to pH 6–7 and incubated at 25° for 18 hr

Table 1. Comparative thin-layer chromatographic* and diagnostic mass spectral† features of microsomal metabolites and derivatives

Compound	R_f	*m/z* (Relative abundance)‡			
		M^+	M-side chain		$CH_2{=}N^+$ <Et(H) / Et>
CLO	0.73	405(5), 407(2)	306(5), 308(3)		86(100)
Desethyl E-CLO	0.59	377(29), 379(13)	306(18), 308(7)		58(100)
C-1	0.59	377(16), 379(5)	306(35), 308(12)		58(100)
3-Hydroxy CLO	0.45				
4-Hydroxy CLO	0.39	421(5), 423(2)	322(3), 324(2)		86(100)
C-2	0.39	421(7), 423(3)	322(5), 324(3)		86(100)
		M-16§	M-N	M-16-side chain	Et_2NOH^+
E-CLO *N*-oxide	0.16	405(35), 407(16)	332(49), 334(37)	306(50), 308(38)	89(36) 86(100)
C-3	0.16	405(37), 407(13)	332(48), 334(33)	306(47), 308(21)	89(40) 86(100)

* Carried out using Analtech 5 × 20 cm (0.25 mm thickness) silica gel GF 254 plates with chloroform–methanol–28% aqueous ammonia (95:5:0.5, v/v) as developing solvent. Spots were visualized under ultraviolet light of 254 nm wavelength.

† Electron ionization mass spectra were recorded on a DuPont model 21-110 spectrometer, except those of C-3 and E-CLO *N*-oxide, which were recorded using a CEC 110B spectrometer. All spectra were recorded at an electron ionization voltage of 70 eV, an ion source temperature of 200–220°, and an average probe temperature of 220°.

‡ Dual entries are listed for ions containing ^{35}Cl, ^{37}Cl.

§ The molecular ion (M^+) was not detected in the spectra of these compounds.

with 2 ml of β-glucuronidase (330 units). The mixture was extracted with 20 ml of ethyl acetate. Comparison of this extract concentrate with that from the control study by TLC [solvent: benzene–triethylamine (90:10, v/v)] indicated no unique components in the former extract. The pooled feces was homogenized, stirred with 250 ml of methanol for 5 hr, and centrifuged at 400 *g* for 15 min. The solution was concentrated *in vacuo*, and the residue was chromatographed on 10 g of 60–200 mesh silica gel. Elution with 200 ml of chloroform–methanol–28% aqueous ammonia (95:5:0.5, v/v) followed by concentration of the eluent gave a yellow residue. This was subjected to preparative TLC using the same solvent as above. E-CLO and other standard compounds (Table 1) were spotted separately on this plate for comparison purposes. A zone with R_f equal to that of 4-hydroxy CLO was present, but none with R_f values equal to the other standards appeared. In some cases, repeated preparative TLC under conditions described above was necessary to afford chromatographically pure material. The metabolite zone was removed from the plate and eluted with 15 ml of ethanol. The extract was centrifuged, and the supernatant fraction was concentrated *in vacuo* (40°) and subjected to chromatographic analysis as summarized in Table 2.

Preparation of benzyl-4-hydroxyphenyl ketone methoxyethoxymethyl ether. In a 250-ml three-necked flask maintained under dry nitrogen, sodium hydride (1.2 g, 50% mineral oil suspension, 26 mmoles) was washed with two 25-ml portions of hexane. Then a solution of benzyl-4-hydroxyphenyl ketone (5.0 g, 24 mmoles) in 30 ml of dry tetrahydrofuran was added dropwise with stirring and cooling. After evolution of hydrogen ceased, a solution of methoxyethoxymethyl chloride (3.6 g, 29 mmoles) in 30 ml of dry tetrahydrofuran was added over a 30-min period to the dark brown reaction suspension. After completion of addition, the flask was allowed to warm to room temperature and, after stirring another 30 min, the beige-colored reaction mixture was shaken with 50-ml portions of benzene and water. The organic extract was further washed with 30 ml of 10% NaOH and three 30-ml portions of water. The organic phase was dried (Na_2SO_4) and concentrated *in vacuo* at 40°. The product was distilled (b.p. 189–191°, 0.15 mm Hg) to give a colorless oil which solidified on standing: NMR ($CDCl_3$) δ 3.30 (s, 3H, OCH_3), 3.38–3.62 (m, 2H, CH_2OCH_3), 3.66–3.90 (m, 2H, OCH_2OCH_2), 4.18 (s, 2H, CH_2Ph), 7.02 (d, J = 9 Hz, 2H, *o*-ArO), 7.22 (s, 5H, C_6H_5), 7.95 (d, J = 9 Hz, 2H, *o*-ArC = O).

Preparation of 4-hydroxy CLO. The above compound was allowed to react with the Grignard reagent prepared from *p*-(2-diethylaminoethoxy)-bromobenzene under previously described conditions [17, 18] which after acid-catalyzed dehydration furnished the deschlorotriarylethylene in 53% yield. Reaction of 1.6 g of this with *N*-chlorosuccinimide was carried out as previously described [14]. The resulting solution of 4-hydroxyclomiphene 2-methoxyethoxymethyl ether in dry chloroform was cooled in ice and treated with HCl gas. After 30 min the mixture was concentrated and the pink residue was dissolved in 25 ml of methanol, adjusted to pH 9 (pHydrion paper) with aqueous sodium carbonate. Then 50 ml of water was added and the mixture was extracted with two 50-ml portions of chloroform. The combined organic extracts were washed with three 50-ml portions of water, dried (Na_2SO_4), and concentrated *in vacuo*. The residue (1.7 g) was chromatographed on 75 g of silica gel. Elution with benzene–triethylamine (20:1, v/v) afforded 0.6 g (43%) of the desired compound which was crystallized from benzene–hexane (5:1, v/v) to give a white powder, m.p. 173–176°. A previously prepared sample of this compound, determined from NMR spectral studies to be an 83:17 mixture of respective E- and Z-isomers, had a melting point of 175–178° [14]. Quantitative chromatographic properties of the present product, determined as summarized in Table 2, were nearly identical to those of the previous one.

Estrogen receptor binding assay. The [^{3}H]estradiol (58 Ci/mmole) used in this assay was obtained from the Amersham Corp. (Arlington Heights, IL); radiochemical purity was checked by TLC. Uteri from Sprague–Dawley rats (200–250 g) were homogenized 1 uterus/2 ml) in ice-cold 10 mM Tris buffer, pH 7.4, which contained 1.5 mM EDTA and

Table 2. Chromatographic comparison of synthetic 4-hydroxy CLO and that produced from E-CLO in the rat

Compound	TLC* R_f value	HPLC† Retention time
Synthetic 4-hydroxy CLO:	(% composition)	
E-isomer	0.30 (81)	13 min (85)
Z-isomer	0.35 (19)	16 min (15)
Rat fecal metabolite	0.30 (70)	13 min
	0.35 (30)	

* Determined using EM 60 silica gel GF 254 TLC plates (plastic backed), 5 × 20 cm (0.20 mm thickness).

The developing solvent was benzene–piperidine (9:1, v/v.) Zones corresponding to separated isomers were visualized under ultraviolet light of 254 nm wavelength, removed and eluted with ethanol, and absorbance was read at 303 nm.

† High performance liquid chromatography (HPLC) was carried out using a 4.6 mm i.d. × 25 cm column packed with 10-μm reversed phase (C_{18}) silica gel (Alltech). The mobile phase (1 ml/min) was 5 mM ammonium acetate buffer, pH 4.5, in methanol–water (77:23 v/v). The instrument was a Waters ALC 202 high performance liquid chromatograph with fixed wavelength detection at 254 nm.

3 mM sodium azide (TEA buffer). The homogenate was centrifuged at 100,000 *g* for 1 hr at 4°. Incubation mixtures contained 200 μl aliquots of the supernatant fraction, 10 μl of a solution of 1.1×10^{-7} M [^{3}H]estradiol in *N,N*-dimethylacetamide, and 10 μl of unlabeled competitor in *N,N*-dimethylacetamide–TEA buffer (1:1). Six concentrations of competitor were used ranging from 1×10^{-10} M to 1×10^{-5} M. Control incubations contained 10 μl of solvent alone, and nonspecific binding was determined in similarly prepared incubations which contained 1×10^{-5} M estradiol. Incubations were performed in triplicate, in 5 ml polypropylene centrifuge tubes, for 2 hr. Then a suspension of 400 μl of dextran-coated charcoal [0.1% dextran (Sigma No. D-1390), 1% acid-washed Norit A in TEA buffer] was added, and the incubation continued for 15 min at 2–4°. Tubes were then centrifuged at 1000 *g* for 10 min, and 400 μl aliquots were dissolved in 5 ml of Scintiverse (Fisher). Bound [^{3}H]estradiol was determined by liquid scintillation spectrometry. Quench corrections were made by the external standard method.

Uterotropic assay for estrogenic activity. Immature Sprague–Dawley female rats (20 to 24-days-old) were obtained from The Holtzman Co., Madison, WI. They were divided randomly into groups of seven animals. To 0.1-ml aliquots of fresh solutions of estradiol benzoate (0.25 mg/ml) and of 4-hydroxy CLO (1, 5, and 25 mg/ml) in *N,N*-dimethylacetamide was added 4.9 ml of peanut oil. The resulting solutions (0.1 ml) were administered s.c. once daily for 3 days. Control animals received vehicle alone. On day 4, the animals were killed by decapitation. The uteri were dissected, and fat and connective tissue were removed. After blotting lightly to remove intraluminal fluid, uteri were weighed to the nearest 0.1 mg. Body weights were also recorded.

Uterotropic assay for antiestrogenic activity. This was carried out exactly as described above, except that animals receiving the 4-hydroxy CLO solutions also received 0.5 μg/0.1 ml of estradiol benzoate, administered separately at different injection sites. One group of control animals each received 0.5 μg of estradiol benzoate, and vehicle; animals in the other group each received two injections of vehicle.

RESULTS

Characterization of synthetic 4-hydroxy CLO. This was prepared in a way similar to that used previously [14], except that the phenolic hydroxyl group of the deoxybenzoin precursor was protected as a 2-methoxyethoxymethyl (MEM) ether, rather than as a tetrahydropyranyl ether. This resulted in a better yield of the intermediate deschlorotriarylethylene, whose carbinol precursor could be dehydrated under dilute acidic conditions without loss of the MEM group [19], thus eliminating the need for re-protection prior to chlorination. Deprotection of the final product was carried out with anhydrous HCl in chloroform, a modification of the method reported for cleavage of MEM ethers of amino phenols [20].

Chromatographic studies. Thin-layer chromatograms of extracts from 30-min incubations of CLO with microsomes exhibited three components not seen in extracts from incubation mixtures in which NADPH was absent. These components (C-1–C-3) were purified and subjected to TLC and mass spectral comparison with standard compounds as summarized in Table 1. These components were, in turn, chromatographically homogeneous with the listed standard compounds of identical R_f value. This was also the case when benzene–triethylamine (90:10, v/v) was used as developing solvent. However, C-2 and C-3 were distinguishable from 3-hydroxy CLO and nicotinamide, respectively, the latter of which was found in extracts from all incubation mixtures in which cofactor was present.

Treatment of either metabolite C-3 or authentic E-CLO *N*-oxide with triphenylphosphine in benzene at 70° for 10 min gave products which were chromatographically indistinguishable from CLO. Deoxygenation of aliphatic *N*-oxides with this reagent has been reported [21].

Estimation of the amounts of C-1–C-3 produced in standard incubation mixtures was made by quantitation of the ultraviolet absorbance at the λ_{max} of each of the respective standards. From these, average recoveries of 7 nmoles of C-1, 12 nmoles of C-2, and 30 nmoles of C-3 were obtained.

Analysis by TLC of urine sample extracts from rats administered E-CLO orally revealed no metabolites or unmetabolized drug. This was also the case with processed fecal extracts, except that a component with R_f approximately equal to that of 4-hydroxy CLO was present. This material was further analyzed for isomeric composition by HPLC and by TLC using a solvent system previously found suitable for separation of geometric isomers of 4-hydroxytriarylethylenes [13]. As suggested by the chromatographic data summarized in Table 2, only the E-isomer was detectable by the former method, but both isomers were observed by the latter one. This discrepancy may have been due to isomerization of this compound on activated silica gel, as was the case in attempted TLC analysis of other 4-hydroxytriarylethylenes [18].

Mass spectral studies. The position and intensity of most peaks in the mass spectra of C-1–C-3 were identical to those in the spectra of the corresponding standard compounds, diagnostic features of which are listed in Table 1. The spectra of C-1 and C-2 exhibited significant molecular ions (M) accompanied by amplified M + 2 ions due to the presence of chlorine substituents. This was also the case for chlorotriaryl ions resulting from side chain fragmentation.

The electron ionization mass spectra of C-3 and E-CLO *N*-oxide did not feature detectable molecular ions but, rather, appeared similar to that of E-CLO. This presumably was due to the ease by which many aliphatic *N*-oxides lose an oxygen atom upon electron impact [22, 23]. In addition, the spectra featured peaks at *m/z* 332(334) and 89. Based on previous studies of structurally related compounds [23, 24], these are due to *N*-oxide rearrangement [25], resulting in formation of a vinyl ether and *N,N*-diethylhydroxylamine. No peak at 393(395), suggestive of alternative *N*-oxide rearrangement with loss of

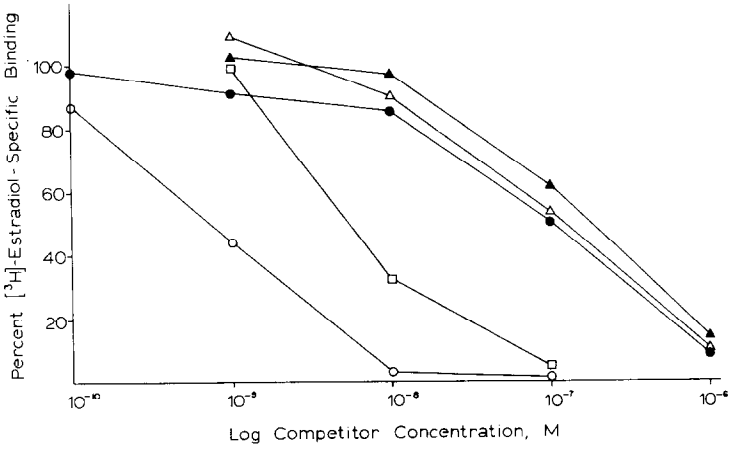

Fig. 1. Effect of increasing concentrations of 4-hydroxy CLO (○), the E-isomers of CLO (●), desethyl CLO (△), and CLO *N*-oxide (▲), and estradiol (□) on the binding of [^{3}H]estradiol to uterine cytosol from mature rats. Incubations were run at 25° for 2 hr. Specifically bound radioactivity (dpm) is plotted as a percentage of that in control incubations. The effect of estradiol at 4° was similar to that shown here.

ethylene, was present. In contrast to these results, the fast atom bombardment mass spectra of C-3 and E-CLO *N*-oxide featured predominant molecular ions (MH^+) at *m/z* 422.

Bioactivity studies. The ability of 4-hydroxy CLO and the E-isomers of CLO, its *N*-oxide and its *N*-desethyl derivative to compete with [^{3}H]estradiol for cytoplasmic estrogen receptors from mature rat uterus was determined by adaption of the dextran-coated charcoal absorption technique [26–28]. Comparative data were also obtained for estradiol. Salient results are summarized in Fig. 1. Relative binding affinities (RBA) calculated from these data are listed in Table 3, in comparison with RBA data from studies carried out at 4°. A study of the uterotropic and antiuterotropic effects of 4-hydroxy CLO was carried out using the 3-day immature uterine weight test [5, 29]. Effects of increasing doses of this compound on uterine weight gain and on the ratio of uterine weight to whole body weight, when given with and without estradiol benzoate, are shown in Fig. 2.

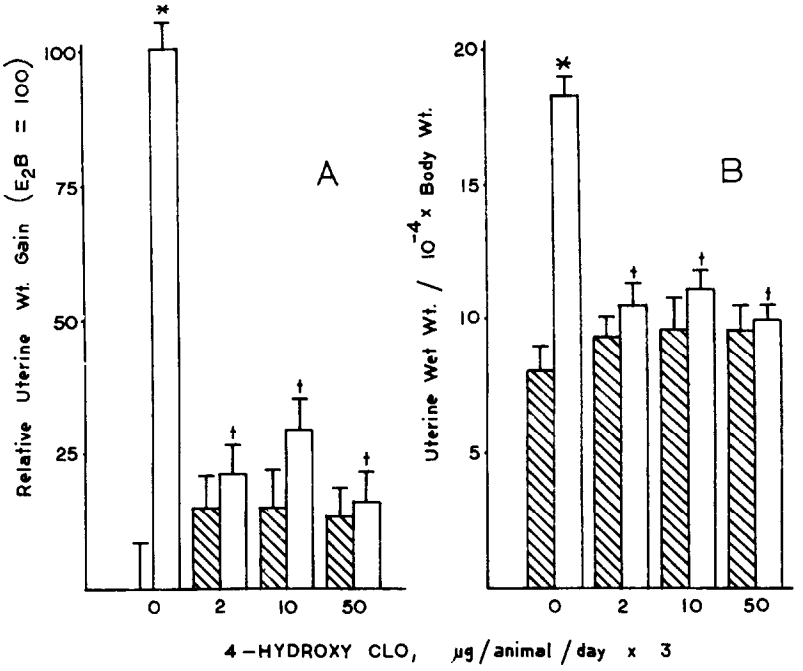

Fig. 2. Uterotropic effect of increasing doses of 4-hydroxy CLO administered without (▨) and with (□) 0.5 µg daily of estradiol benzoate (E_2B) on uterine weight gain (A), and the ratio of uterine wet weight to body weight (B), in the 3-day immature rat uterotropic assay. The average uterine wet weight from animals receiving vehicle only was 75 mg; that from those receiving 0.5 µg daily of E_2B plus vehicle was 185 mg. There were seven animals per treatment group. Standard errors are indicated by brackets. Results which differed significantly from vehicle-treated and E_2B-treated controls ($P < 0.05$) are indicated, in turn, by asterisks (*) and daggers (†).

Table 3. Influence of incubation temperature on affinity of E-CLO and analogues for rat uterine cytosol receptor*

Compound	Relative binding affinity†	
	4°	25°
4-Hydroxy CLO	331	757
E-CLO *N*-oxide	0.88	2.9
E-CLO	0.71	5.3
E-Desethyl CLO	0.62	3.7

* The competitive binding assay employed cytosol from mature rat uteri, 5×10^{-9} M ^{3}[H]estradiol, 1×10^{-10} to 1×10^{-5} M unlabeled competitor, and conditions exactly as described in Materials and Methods.

† This was determined from the concentration of unlabeled estradiol required to inhibit 50% of the specific [^{3}H]estradiol binding (5×10^{-9} M) divided by the concentration of E-CLO and analogues required to do the same. The relative binding affinity of estradiol was set at 100.

DISCUSSION

For a compound to have estrogenic and/or antiestrogenic activity, binding to cytosol estrogen receptors is thought to be a requirement [27, 30]. Furthermore, in triarylethylenes capable of geometric isomerism, *trans*-isomers (e.g. E-CLO) are bound more strongly than are the respective *cis*-isomers [31]. Thus, we determined the affinities of E-CLO and configurationally analogous synthetic standards of all of its metabolites except 4-hydroxy CLO. This compound was composed of about an 80:20 mixture of E-4-hydroxy CLO and its geometric isomer. As indicated in Fig. 1 and Table 3, this last compound was substantially more strongly bound than were the first three. Affinity of all compounds was greater at 25° than at 4°. These results are qualitatively consistent with those obtained with 4-hydroxy TAM and a related phenolic triarylethylene under similar conditions [28, 32], but they differ from those obtained with TAM and desmethyl TAM which had decreased affinity at the higher temperature [28].

The RBA of 4-hydroxy CLO was somewhat greater then that of 4-hydroxy TAM or desmethyl NIT, which had similarly determined RBAs (4°) of 213 and 135 respectively [12, 18]. These RBA differences would not be expected to be due to differences in side chain structure and basicity, based on a previous study of the relationship of structure (basicity) to estrogen receptor affinity in a series of TAM analogues [33]. Rather, these differences may be due to differences in hydroxyl group acidity. These compounds are, in turn, vinylogues* of 4-chlorophenol (pK_a 9.18), 4-ethylphenol (pK_a 10.00), and 4-nitrophenol (pK_a 7.15). Thus, the acidity of the hydroxyl in 4-hydroxy CLO is intermediate to those of the other two compounds and may more closely approach an optimum required for maximal estrogen receptor affinity. But to establish more clearly the influence of phenolic acidity on cytosol estrogen receptor affinity of 4-hydroxytriarylethylenes, comparative binding studies of configurationally similar analogues with structural features affording a greater array of acidities will be needed.

The microsomal studies suggest that CLO underwent metabolism via *N*-dealkylation, 4-hydroxylation, and *N*-oxide formation, results which are qualitatively similar to those obtained from incubation of TAM with phenobarbital-induced rat microsomes [24]. In contrast, under identical experimental conditions using rabbit microsomes, *N*-oxide formation was not evident with TAM or the isomers of CLO as substrates ([14], unpublished results).

Earlier experimental and clinical studies indicated that CLO was eliminated mainly in the feces [34]. Although our analytical method was not of sufficient selectivity and sensitivity to make final conclusions about the fecal elimination of CLO, the preliminary results reported here suggest that this was largely as 4-hydroxy CLO. A similar metabolic fate has been reported for TAM in several species including man, in which 4-hydroxy TAM was the major fecal metabolite accompanied by lesser amounts of a variety of other metabolites [35, 36].

Results of the *in vivo* metabolism and estrogen receptor binding studies prompted us to determine the estrogenic and antiestrogenic effects of 4-hydroxy CLO in the 3-day immature rat uterotropic assay. As shown in Fig. 2A, administration of 4-hydroxy CLO by itself had a modest estrogenic effect which was maximal at a dose of 2 μg/animal. Antagonism of the uterotropic effect of E_2B, by about 75%, was also maximal at this dose. Data in Fig. 2B indicated that these effects were independent of overall weight gain. Thus, 4-hydroxy CLO appears to be a more potent antiestrogen in this assay than is E-CLO, since the latter had maximal activity (67% antagonism) at a dose of 16 μg/animal [5].

Although 4-hydroxy CLO had potent antiestrogenic activity and was a major elimination product, the significance of its contribution to the bioactivity of CLO remains to be established. Analysis of plasma of patients receiving clomiphene therapy revealed the presence of unchanged CLO and lesser amounts of several as yet unidentified metabolites [37]. Similar studies with TAM have shown 4-hydroxy TAM to be present in relatively low plasma concentration in comparison with levels of unchanged drug, desmethyl TAM [38, 39], and other side-chain-altered metabolites, which could contribute to bioactivity [40, 41]. On the other hand, phenolic metabolites of triarylethylenes are accumulated in estrogen-receptor-containing tissues [12, 13, 42–44], suggesting that plasma levels of these drugs and their respective metabolites may not be a completely accurate relfection of their contribution to bioactivity. Further studies of plasma and target tissue levels of CLO and metabolites should help to define the role of 4-hydroxy CLO and other metabolites in modulating the estrogenic and antiestrogenic effects of CLO.

* Because of the resonance effect transmitted through the vinyl group (carbon–carbon double bond), the acidities of these compounds should parallel these of the corresponding 4-substituted phenols: J. R. Johnson, in *Organic Chemistry: An Advanced Treatise* (Ed. H. Gilman), 2nd Edn, Vol. 2, p. 1909. John Wiley, New York (1943).

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REFERENCES

1. V. C. Jordan, C. J. Dix, K. E. Naylor, G. Prestwich and L. Rowsby, *J. Toxic. environ. Hlth.* **4**, 363 (1978).
2. L. C. Huppert, *Fert. Steril.* **31**, 1 (1979).
3. A. J. W. Hsueh, G. F. Erickson and S. S. C. Yen, *Nature, Lond.* **273**, 57 (1978).
4. E. Y. Adashi, A. J. W. Hsueh, T. H. Bambino and S. S. C. Yen, *Am. J. Physiol.* **240**, E125 (1981).
5. V. C. Jordan, B. Haldemann and K. E. Allen, *Endocrinology* **108**, 1353 (1981).
6. E. Kokko, O. Jänne, A. Kauppila and R. Vihko, *J. clin. Endocr. Metab.* **52**, 345 (1981).
7. R. L. Sutherland and L. C. Murphy, *Molec. cell. Endocr.* **25**, 5 (1982).
8. K. D. Schultz, B. Haselmayer and F. Holzel, in *Basic Action of Sex Steroids on Target Organs* (Eds. P. O. Hubinont, F. Leroy and P. Galand), pp. 274–9. Karger, Basel (1971).
9. J. J. Li, T. L. Cuthbertson and S. A. Li, *J. natn. Cancer Inst.* **64**, 795 (1980).
10. E. Hecker, I. Vegh, C. M. Levy, C. A. Magin, J. C. Martinez, J. Loureiro and R. E. Garola, *Eur. J. Cancer* **10**, 747 (1974).
11. S. S. Legha and S. K. Carter, *Cancer Treat. Rev.* **3**, 205 (1976).
12. J. R. Hayes, E. A. Rorke, D. W. Robertson, B. S. Katzenellenbogen and J. A. Katzenellenbogen, *Endocrinology* **108**, 164 (1981).
13. D. W. Robertson, J. A. Katzenellenbogen, D. J. Long, E. A. Rorke and B. S. Katzenellenbogen, *J. Steroid Biochem.* **16**, 1 (1982).
14. P. C. Ruenitz, *Drug Metab. Dispos.* **9**, 456 (1981).
15. S. A. Martin, C. E. Costello and K. Biemann, *Analyt. Chem.* **54**, 2362 (1982).
16. P. C. Ruenitz and M. M. Toledo, *Biochem. Pharmac.* **30**, 2203 (1981).
17. D. J. Collins, J. J. Hobbs and C. W. Emmens, *J. med. Chem.* **14**, 952 (1971).
18. P. C. Ruenitz, J. R. Bagley and C. M. Mokler, *J. med. Chem.* **25**, 1056 (1982).
19. E. J. Corey, J-L. Gras and P. Ulrich, *Tetrahedron Lett.* 809 (1976).
20. J. Quick and R. Ramachandra, *Synth. Commun.* **8**, 511 (1978).
21. L. Horner and H. Hoffmann, *Angew. Chem.* **68**, 473 (1956).
22. J. K. Faulkner and K. J. A. Smith, *J. Pharm. Pharmac.* **26**, 473 (1974).
23. I. Bornschein, A. Gehrke, S. Pfeifer and R. Kraft, *Pharmazie* **32**, 695 (1977).
24. A. B. Foster, L. J. Griggs, M. Jarman, J. M. S. van Maanen and H-R. Schulten, *Biochem. Pharmac.* **29**, 1977 (1980).
25. A. C. Cope and C. L. Bumgardner, *J. Am. chem. Soc.* **79**, 960 (1957).
26. V. C. Jordan, M. M. Collins, L. Rowsby and G. Prestwich, *J. Endocr.* **75**, 305 (1977).
27. E. R. Ferguson and B. S. Katzenellenbogen, *Endocrinology* **100**, 1242 (1977).
28. A. E. Wakeling and S. R. Slater, *Cancer Treat. Rep.* **64**, 741 (1980).
29. R. W. Hartmann, G. Kranzfelder, E. von Angerer and H. Schönenberger, *J. med. Chem.* **23**, 841 (1980).
30. B. S. Katzenellenbogen and E. R. Ferguson, *Endocrinology* **97**, 1 (1975).
31. J. R. Skidmore, A. L. Walpole and J. Woodburn, *J. Endocr.* **52**, 289 (1972).
32. L. J. Black, C. D. Jones and R. L. Goode, *Molec. cell. Endocr.* **22**, 95 (1981).
33. D. W. Robertson, J. A. Katzenellenbogen, J. R. Hayes and B. S. Katzenellenbogen, *J. med. Chem.* **25**, 167 (1982).
34. E. Schreiber, J. E. Johnson, E. J. Plotz and M. Wiener, *Clin. Res.* **14**, 287 (1966).
35. J. M. Fromson, S. Pearson and S. Bramah, *Xenobiotica* **3**, 693 (1973).
36. J. M. Fromson, S. Pearson and S. Bramah, *Xenobiotica* **3**, 711 (1973).
37. P. J. Harman, G. L. Blackman and G. Phillipou, *J. Chromat.* **225**, 131 (1981).
38. H. K. Adam, J. S. Patterson and J. V. Kemp, *Cancer Treat. Rep.* **64**, 761 (1980).
39. C. Fabian, L. Tilzer and L. Sternson, *Biopharmac. Drug Dispos.* **2**, 381 (1981).
40. R. R. Bain and V. C. Jordan, *Biochem. Pharmac.* **32**, 373 (1983).
41. D. J. Bates, A. B. Foster, L. J. Griggs, M. Jarman, G. Leclercq and N. Devleeschouwer, *Biochem. Pharmac.* **31**, 2823 (1982).
42. B. S. Katzenellenbogen, E. J. Pavlik, D. W. Robertson and J. A. Katzenellenbogen, *J. biol. Chem.* **256**, 2908 (1981).
43. V. C. Jordan and R. A. Bowser-Finn, *Endocrinology* **110**, 1281 (1982).
44. J-L. Borgna and H. Rochefort, *J. biol. Chem.* **256**, 859 (1981).