Biochemical Pharmacology, Vol. 55, pp. 841–851, 1998.
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ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00574-1

Clomiphene Analogs with Activity *In Vitro* and *In Vivo* against Human Breast Cancer Cells

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ABSTRACT. Six hundred triphenylethylenes were assayed for antiproliferative activity against MCF-7, LY2, and MDA-MB-231 breast cancer cells using sulforhodamine B dye to measure proliferation. Here we report on just 63 of the compounds, mostly clomiphene analogs, with substitutions on the α′ or β ring, at the vinyl position or in the side chain, of which 23 were active, as defined by antiproliferation IC_{50} values ≤1 μM. Activity profiles showed that 23 and 11 analogs were active toward MCF-7 and LY2, respectively, but none were active against MDA-MB-231. The IC_{50} values of tamoxifen were 2.0 μM against MCF-7 and 7.5 μM against LY2 and MDA-MB-231. Estradiol reversed antiproliferative activities of several *E* isomers but not their *Z* isomer counterparts. Clomiphene side chain analogs **46** [(*E*)-1-butanamine, 4-[4-(2-chloro-1,2-diphenylethenyl) phenoxy]-*N*,*N*-diethyl-dihydrogen citrate (MDL 103,323)] and **57** [(*E*)-*N*-[*p*-(2-chloro-1,2-diphenylvinyl) phenyl]-*N*,*N*-diethylethylenediamine dihydrogen citrate (MDL 101,986)] were 4- to 5-fold more effective than tamoxifen. Methylene additions up to $(-CH_2-)_{12}$ in the clomiphene side chain showed that analog **46** [$(-CH_2-)_4$ side chain] had maximal antiproliferative activity, binding affinity, and inhibition of transcription of an estrogen response element luciferase construct in transfected MCF-7 cells. Intraperitoneal administration of **46** or **57** inhibited progression of MCF-7 breast tumor xenografts in nude mice with ED_{50} values of <0.02 mg/mouse/day. Both analogs may hold promise for treating ER positive breast cancer and are of interest for further development. BIOCHEM PHARMACOL **55**;6:841–851, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. clomiphene analogs; antiestrogens; breast cancer; MCF-7; LY2; tamoxifen resistance; estrogen receptor

The number of new cases of breast cancer diagnosed every year is approximately 180,000, making this disease the most prevalent cancer in women [1]. Breast cancer is a hormone-dependent cancer, and an estimated 70% of tumors are positive for the ER‖ [2]. Two classes of antiestrogen drugs known to antagonize the growth of hormone-dependent breast cancer cells are steroidal antiestrogens such as ICI 164,384 [3] and nonsteroidal antiestrogens such as the TPEs clomiphene [4], toremifene [5], and tamoxifen [6, 7]. Tamoxifen is used worldwide by over a million women for the treatment of ER positive breast cancer. Its continued use has reduced both the annual rate of death and disease recurrence among breast cancer patients [8], and it has a low incidence of short- and long-term side-effects. However, tamoxifen resistance eventually develops, resulting in the failure of tamoxifen therapy, thus creating the need for additional nontoxic therapeutic modalities.

Prior to the development of tamoxifen, the first reported active nonsteroidal antiestrogen, ethamoxytriphetol (MER-25), was synthesized at the Wm. S. Merrell Co. [9] and subsequently shown to have antifertility activity [10] and antitumor activity [11]. These findings were the incentive for more intensive efforts leading to the synthesis of clomiphene [12], Upjohn's nafoxidine [13], and later tamoxifen at Imperial Chemical Industries [14]. For a review of antiestrogens, see Ref. 15. Clomiphene is marketed by Hoechst Marion Roussel as a fertility agent, but it has been used in clinical trials for the treatment of breast cancer [16]. Cumulative results were published from various clinical trials, and objective responses were noted in 28% of late stage breast cancer patients given clomiphene, which compared favorably with responses to tamoxifen (27%) in unselected patients [17]. Because of our earlier efforts in antiestrogen research, approximately 600 TPEs were avail-

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‖ *Abbreviations:* CSCS, charcoal stripped calf serum; ER, estrogen receptor; FBS, fetal bovine serum; HBSS, Hanks' Balanced Salt Solution; IMEM, Improved Minimum Essential Medium, Eagle's; MTG, monothioglycerol; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSB, nonspecific binding; RBA, relative binding affinity; SRB, sulforhodamine B dye; tam, tamoxifen citrate; and TPE, triphenylethylene.

Received 21 January 1997; accepted 12 September 1997.

able in the Hoechst Marion Roussel chemical inventory. Having set goals to seek compounds significantly more potent than tamoxifen, and with efficacy against tamoxifen-resistant breast cancer cells, we screened the TPEs for antiproliferative activity against three human breast cancer cell lines: MCF-7 (ER positive, tamoxifen-susceptible), LY2 (ER positive, tamoxifen-resistant), and MDA-MB-231 (ER negative, tamoxifen-resistant).

In this paper, we present results on 63 TPEs with substitutions in the α ring side chain, or on the α′ or β ring, or at the vinyl Cl of clomiphene. Of the 63 analogs, 23 were found to have antiproliferative IC_{50} values ≤1 μM, and many had IC_{50} values lower than that of tamoxifen toward MCF-7 and LY2. Furthermore, several of the 23 analogs were separated into pure *E* and *Z* isomers to define isomer specificities in terms of biological and biochemical activities including antiproliferative assays, antitumor activity in nude mice, and ER relative binding activities.

MDL 103,323 [(*E*)-1-butanamine, 4-[4-(2-chloro-1,2-diphenylethenyl)phenoxy]-*N*,*N*-diethyl-dihydrogen citrate] and MDL 101,986 [(*E*)-*N*-[*p*-(2-chloro-1,2-diphenylvinyl)phenyl]-*N*,*N*-diethylethylenediamine dihydrogen citrate] are *E* isomers of the clomiphene analogs **46** and **57**, respectively. Both showed significant antitumor activity against MCF-7 human tumor xenografts in nude mice with ED_{50} values <0.02 mg/mouse/day, administered orally for 6 weeks. In addition, the antiproliferative activities of both isomers were several-fold better than tamoxifen toward MCF-7 and LY2 cell lines. The biological activities of these analogs suggest potential utility for the treatment of estrogen-dependent breast cancer.

MATERIALS AND METHODS

Cell Lines

MCF-7 (ATCC HTB 22) and MDA-MB-231 (ATCC HTB 26) were obtained from the American Type Culture Collection. LY2 cells were provided by Dr. Marc Lippman [18]. Cells were maintained in Costar T75 flasks containing IMEM without phenol red (Biofluids, Inc.) supplemented with 4 mM glutamine and 5% FBS (Gibco BRL).

TPEs

All compounds were analyzed for structural integrity and spectral purity by NMR prior to their use in assays. The majority of TPEs had been synthesized as mixtures of isomers, and were screened without further modifications. However, several of the isomer mixtures that showed interesting biological activity were resolved into pure isomers and retested. To obtain pure *E* and *Z* isomers, isomeric mixtures were made basic with 2 N NaOH, extracted with chloroform, and analyzed by HPLC on a Porasil column (Waters) monitored by UV at 270 nm. The mobile solvent was hexane:chloroform:triethylamine (20:80:0.02). For preparative HPLC, a 19 × 300 mm semipreparative column was used with a flow of 15 mL/min and an injection volume of 50 μL containing 25 mg of compound. The individual isomer peaks were collected and identified by NMR and mass spectrometry. The *E* isomer of **22** (4-hydroxyclomiphene) isomerized to an approximately equal mixture of *E* and *Z* isomers within 2 weeks in hexane/$CHCl_3$, but not in DMSO. Binding assays with the *E* isomer were performed within 3 days of its dissolution in DMSO. Tamoxifen citrate (tam) and 4-hydroxytamoxifen citrate were obtained from ICI America, Inc.

Antiproliferation Assay

SRB stains protein and is used to measure cell growth. Because the SRB assay is suitable for large-scale screening with several practical advantages over the MTT assay, the National Cancer Institute adopted this assay for use in routine antiproliferative screening.

Antiproliferative assays were performed using SRB (Sigma) as described [19] with modifications. Cells were harvested when nearly confluent from IMEM/FBS using trypsin/EDTA, washed once with serum-free IMEM, and resuspended in IMEM/FBS. Stock drug solutions (10 mM) were prepared in DMSO and diluted with serum-free IMEM. Drug dilutions and all additions of drugs, cells, and medium to microtiter wells were made with a Perkin Elmer Cetus PRO/PETTE. Aliquots (100 μL) of 1×10^4 MCF-7 cells or 3×10^3 LY2 or MDA-MB-231 cells were dispensed in duplicate into 96-well microtiter plates and incubated at 37° in 5% CO_2 for 20–24 hr, and the medium was replaced with 100 μL of IMEM/FBS containing drug concentrations from 0.078 to 10 μM in duplicate. After 4 days of incubation, the medium and drugs were replaced. After a total of 8 days of incubation, the medium was removed and the cell monolayers were fixed for 60 min at 4° with 100 μL of 10% trichloroacetic acid, rinsed five times with water, and dried. The fixed cells were stained for 30 min at room temperature with 100 μL of 0.4% SRB in 1% acetic acid, rinsed four times with 1% acetic acid, and dried, and the SRB was extracted for 5 min with 100 μL of 10 mM Tris base, pH 10.5. Absorbances were determined at 492 nm with a Titertek Multiscan MCC/340 plate reader. Concentration–response curves were constructed to estimate IC_{50} values, defined as the micromolar concentration of drug inhibiting 50% of proliferation. To determine the effect of estradiol on IC_{50} values, compounds were assayed in medium supplemented with 0.1 μM estradiol (Sigma).

The following guidelines were used for making comparisons on various compound activities. Active compounds had antiproliferative IC_{50} values ≤1 μM toward any cell line, compounds were selective for MCF-7 or LY2 if antiproliferative IC_{50} values against the cell lines differed by ≥5-fold, and estradiol reversal of growth antagonism was positive if the IC_{50} in estradiol-supplemented medium was ≥3-fold the IC_{50} determined in unsupplemented medium.

Extraction of ER

MCF-7 or LY2 cells were cultured for 15–30 passages in IMEM supplemented with 5% CSCS (Cocalico Biologicals) and 4 μg/mL bovine insulin (Gibco BRL), since preliminary assays indicated the ER yield was 2- to 3-fold greater if CSCS was substituted for FBS. The monolayers were rinsed with HBSS (Gibco BRL), scraped into HBSS containing 0.1% (v/v) MTG, and centrifuged for 10 min at 800 *g*. To extract total ER (cytosolic + nuclear), cells were resuspended in 2 packed cell volumes of high salt extraction buffer [10% (v/v) glycerol, 500 mM KCl, 25 mM HEPES buffer, pH 7.8] [18, 20, 21], frozen and thawed three times, mixed for 30 min at 4°, and centrifuged at 4° for 30 min at 12,000 *g*. Supernatants were retained as the source of ER and stored at −80°.

Relative Binding Affinities

RBAs were determined in 96-well microtiter plates with conical wells [22]. Drugs were prepared as 10 mM stock solutions in DMSO, and further dilutions were made with Tris/EDTA buffer (TE buffer) containing the following supplements and final concentrations of 8 mM Tris, pH 7.4, 1 mM EDTA, 0.4% BSA [23], 12.5% (v/v) dimethylformamide, 0.1% (v/v) MTG and 2 nM [2,4,6,7-^{3}H]estradiol, 114 Ci/mmol (Amersham). Cell extracts (15 μL) were added to begin the assay in final volumes of 100 μL, in triplicate, and incubated at 4° for 16–18 hr. Receptor bound [^{3}H]estradiol was separated from unbound [^{3}H]estradiol with 100 μL of TE buffer, pH 7.4, containing 0.1% (v/v) MTG, 0.5% BSA, 0.05% dextran T70, and 0.5% Norit A at 4° for 15 min and centrifuged at 4° for 20 min at 1200 *g*. The mean net disintegrations per minute were determined in 160 μL of supernatant by subtracting the mean of the NSB (NSB = dpm bound in the presence of 1 μM nonradioactive estradiol). The IC_{50} values were estimated from percent control versus concentration curves, and RBAs were calculated from the expression:

$$\text{RBA} = \frac{IC_{50}\ \text{estradiol}}{IC_{50}\ \text{TPE}} \times 100, \text{ according to Korenman [24].}$$

Concentration–response curves of several *E* isomers were analyzed for parallelism using Graph Pad software (Prism Version 2.01).

Transfection

To determine whether isomers of side chain analogs inhibited expression of an estrogen responsive gene, the pGL 2-basic vector (Promega) was digested with *Sma*I and *Xho*I, and a DNA fragment containing two copies of the vitellogenin estrogen response element [25], adjacent to a 180 bp fragment encoding the thymidine kinase promoter [26], was inserted upstream to the luciferase gene. This plasmid, pVETLUC, was provided by Drs. Steven Busch and Gary Martin. MCF-7 cells were transfected with the pVETLUC plasmid by electroporation. Cells (2×10^6) were combined with 50 μg of plasmid DNA in 1 mL of OPTI-MEM 1 medium in an electroporation chamber (Gibco BRL). The suspension was subjected to a charge of 500 V/cm, 800 microfarads, at 0° and low resistance. Following a 1-min recovery period, the cells were resuspended in growth medium, viability was assessed by trypan blue exclusion, and cells were dispensed into 96-well microtiter plates at approximately 1×10^4 cells/well. The culture medium was replaced with fresh medium after 4 hr of incubation at 37°, and after 24 hr with fresh medium containing 1 nM estradiol and side chain-extended analogs and incubated for 18–22 hr. The cells were rinsed once with HBSS and, after freezing at −70° for 15 min, 150 μL of lysis buffer (Promega) was added and the plates were agitated for 20 min at ambient temperature. The lysates were analyzed for luciferase (Promega assay system) in a luminometer. The IC_{50} values were determined from log-log curve fits using Biolinks software from Dynatech.

Antitumor Effects of TPEs

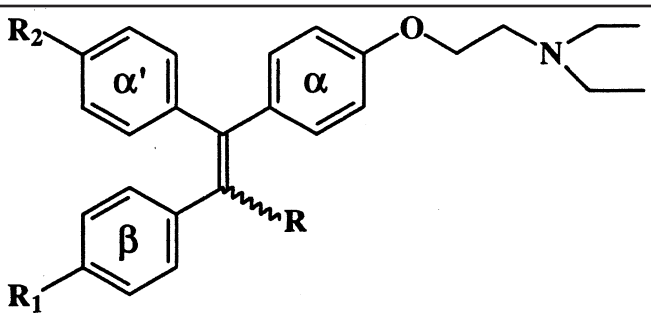
Nude mice were housed in microisolator cages under positive air pressure, and all surgical manipulations and drug treatments were performed in a laminar flow cabinet. MCF-7 cells (2×10^6) were inoculated s.c. into the flanks of female *nu/nu* mice, and tumors were allowed to develop. Tumors of 400–500 mm^3 were taken from maintenance mice, cut into 2-mm^3 pieces, and transplanted into the flanks of naive mice using a 13-Ga trocar. These xenografts were allowed to grow to volumes of 50–100 mm^3, at which time mice (N = 6) were assigned randomly to control or drug treatment groups. To assess tumor growth and the effects of TPEs, tumors were measured weekly with Vernier calipers in two dimensions as described previously [27]. TPEs were administered daily, 5 days/week, as a solution in 6% ethanol, 4% Tween 80, 0.8% NaCl, and 0.68 mM citric acid (0.2 mL/dose) [28].

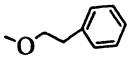
RESULTS

Substitutions on the α′ and β Rings of 1 *and* 15*, and at the Vinyl Cl of* 15

The compounds in Table 1 consist of two groups, analogs of **1**, R = H (**2**–**14**) and analogs of **15**, R = Cl (clomiphene) (**16**–**25**). The necessity of Cl for activity ($IC_{50} \leq 1$ μM, see Materials and Methods) is clearly shown, since compound **15** was ten and five times more active than compound **1** toward MCF-7 and LY2, respectively. Further comparisons show that just 2 analogs of **1** (**7** and **9**) and 6 analogs of **15** (**16**, **19**, and **22**–**25**) were active. Compound **26** was also active, but it is the HCl salt of clomiphene, not a clomiphene analog. Very little improvement in the activity of **15** against MCF-7 was generated by various substitutions at R_1 or R_2 except for the hydroxy analog (**22**) which was 10 times more active than **15** and 100 times more active than

TABLE 1. Antiproliferative activities of analogs substituted on the α′ and β rings of 1 and 15



Compound	R	R_1	R_2	IC_{50}* (μM) MCF-7	LY2	MDA-MB-231
1†	H‡	H	H	8.0	8.0	ND§
2	H	CH_3	H	4.0	4.0	ND
3	H	OCH_3	H	2.0	3.0	4.5
4	H	Cl	H	5.8	6.2	ND
5	H	F	H	1.4	3.3	ND
6	H	$C(CH_3)_3$	H	1.3	4.5	ND
7	H	Biphenyl	H	0.85	3.0	ND
8	H	H	CH_3	3.4	2.5	ND
9	H	OCH_3	CH_3	1.0	2.7	6.0
10	H	Cl	CH_3	4.1	6.2	6.2
11	H	H	Cl	3.2	3.2	ND
12	H	OCH_3	Cl	3.0	3.5	ND
13	H	Cl	Cl	3.0	4.7	ND
14	H	H	O-DEAE‖	2.7	5.4	ND
15	Cl	H	H	0.8	1.5	7.2
16	Cl	OH	H	3.0	1.0	> 10
17	Cl	OCH_3	H	1.3	2.3	6.6
18	Cl	Cl	H	1.5	3.0	4.0
19	Cl	Cl	CH_3	0.5	1.9	3.6
20	Cl	$C(CH_3)_3$	H	1.2	3.3	ND
21	Cl		H	1.1	3.0	ND
22	Cl	H	OH	0.07	1.6	> 10
23	Cl	H	CH_3	0.8	1.0	ND
24	Cl	H	OCH_3	0.6	0.8	6.5
25	Cl	OCH_3	Cl	0.8	0.9	3.1
26	Cl	H	H	0.6	1.4	7.2

* Most IC_{50} values ≤1 μM are means of at least two experiments.
† Compounds are citrate salts except for **1**, **2**, **4**, **7**, **13**, **19**, **25**, and **26** which are hydrochloride salts and **6** which is a free base.
‡ Isomer configuration: isomeric mixtures except for **1**, **4**, **6**, **13**, and **14** are one isomer of unknown configuration; **7** is *E* configuration; **10** and **11**, isomer status unknown.
§ ND = not determined.
‖ $O(CH_2)_2N(C_2H_5)_2$.

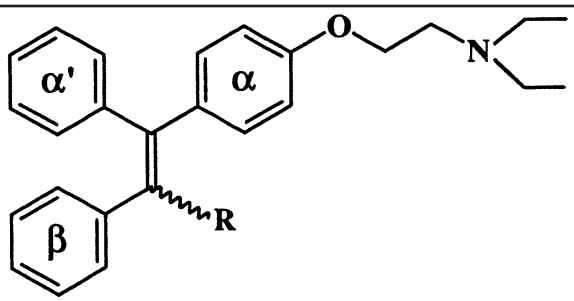
1. Furthermore, compound **22** was about 40 times more active than compound **16** against MCF-7, demonstrating the superior antiproliferative activity of the β-4-OH over the α′-4-OH.

Several analogs of **15** with various substitutions for the vinyl Cl are shown in Table 2. The only improvements in antiproliferative activity seen with these analogs were with compounds **27**, **33**, and **34**, which were at best 2-fold more active than **15** against LY2 cells.

Variations on the Alkyl Ether Side Chain

A number of substitutions were made on the alkyl ether side chain of clomiphene, as shown in Table 3. Compounds **38** and **40** are included for comparison to show that without side chains the structures were devoid of activity against MCF-7 or LY2. The unsubstituted vinyl compound (**39**) is shown for comparison to **51** to emphasize the potency differential between **39** and **51**, that is, **51** was more active than **39** by a factor of 21. In reference to the 17 analogs (**41**–**57**), 11 showed either the same activity as **15**, or were more active than **15**, against MCF-7. Among those were clomiphene side chain analogs that markedly affected antiproliferative activity. For example, variations on the alkylamino groups showed the monoethyl analog of clomiphene (**43**), as well as clomiphene, to be approximately 8-fold more active than the dipropyl analog of clomiphene (**41**). In addition, compound **46**, which differs from **15** by an extension of 2 methylene groups in the side chain producing a butyl chain, was more active than **15**

TABLE 2. Antiproliferative activity of vinyl substituted clomiphene analogs against breast cancer cells



Compound*	R†	IC_{50}‡ (μM)		
		MCF-7	LY2	MDA-MB-231
1§	H	8.0	8.0	ND‖
15§	Cl	0.8	1.5	7.2
27	Br	0.8	0.7	6.4
28	F	2.7	2.3	ND
29	NO_2	2.0	2.0	ND
30	CN	6.0	5.0	ND
31	$CONH_2$	2.5	2.5	ND
32	CH_3	2.4	2.4	ND
33	CH_2CH_3	0.7	0.7	5.4
34	$(CH_2)_2CH_3$	1.0	1.0	5.2
35	$(CH_2)_3CH_3$	2.5	2.5	ND
36	$O(CH_2)_2N(C_2H_5)_2$	6.0	6.0	ND
37	C_6H_5	3.0	3.5	ND

* Compounds are citrate salts except for **33** which is an HCl salt, **36** which is an oxalate salt, and **31** and **37** which are free bases.
† Isomer configurations: isomer mixtures except for **30** and **35** are one isomer of unknown configuration, and the isomer status is unknown for **36**.
‡ IC_{50} values ≤1 μM are means of at least two determinations.
§ Compounds from Table 1 included here for comparison.
‖ ND = not determined.

against both MCF-7 and LY2. Therefore, to investigate the biological effects of longer side chains, additional analogs with side chains extending from 5 to 12 methylene groups were synthesized. *E* and *Z* isomers of the analogs were purified and assayed for antagonism of MCF-7 growth, estradiol-enhanced expression of luciferase in transiently transfected MCF-7 cells and estrodial binding to MCF-7 ER, as shown in Table 4. Maximal activity against cell growth, luciferase expression, and in the competitive binding assay correlated with the *E* isomer of the 4 carbon side chain analog (**46**). Side chain extensions longer than the butyl side chain did not improve activity, and regardless of side chain length the *Z* isomers were uniformly less active.

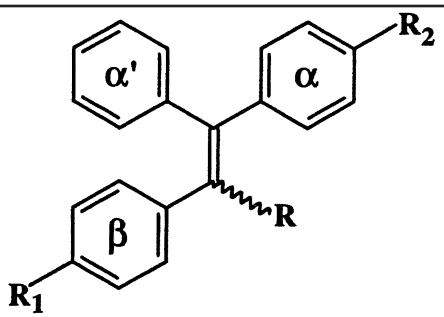
Further observations from Table 3 show that the activity of clomiphene against MCF-7, but not LY2, was either maintained or improved upon by a number of analogs with heterocyclic groups such as pyrrolidyl (**47**), piperidyl (**48**, **51**) and 4-methylpiperazinyl (**50**). Therefore, we synthesized analogs with pyrrolidyl (**52**), piperidyl (**53**) or 4-methylpiperazinyl (**54**) groups and butyl side chains in anticipation that the butyl side chain would enhance antiproliferative activity. However, activities were not improved upon, since the IC_{50} values of the butyl side chain analogs were substantially greater than those of the respective ethyl side chain counterparts. Additional side chain analogs substituting O with C (**55**), S (**56**), or N (**57**) produced activities the same as or 2–3 times greater than that of clomiphene against MCF-7. However, the activities of these analogs against LY2 were the same as or not as potent as clomiphene.

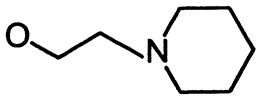
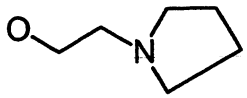
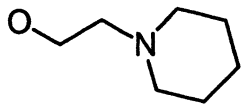
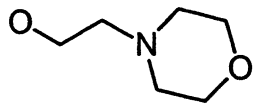
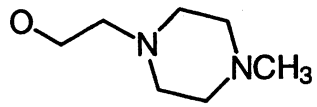
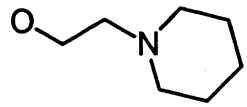
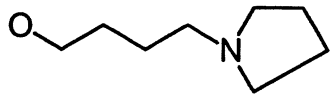
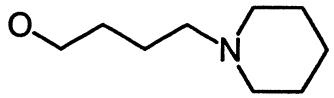
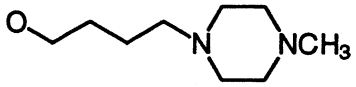
Estradiol Reversal of Activities of Pure Isomers and of 22

Pure *E* and *Z* isomers of several analogs were assayed for cell growth antagonism and reversal by estradiol, as shown in Table 5. While both isomers of each analog elicited some degree of growth antagonism, estradiol reversal was positive if estradiol supplementation increased the IC_{50} by ≥3-fold over the IC_{50} in unsupplemented medium. Thus, growth antagonism by *E* isomers **15**, **23**, **24**, and **27** was reversed in LY2 cells but not in MCF-7 cells. However, growth antagonism by *E* isomers **46**, **48**, **55**, **56**, and **57** was reversed in both cell lines. None of the *Z* isomer-induced growth antagonism was reversed by estradiol.

As mentioned above, compound **22** (4-hydroxyclomiphene) had the lowest IC_{50} of all clomiphene analogs tested against MCF-7. The data in Fig. 1 show a biphasic concentration–response curve of compound **22** mediated growth antagonism and a monophasic concentration response showing reversal by estradiol. Reversal of antagonism was complete at concentrations of **22** up to 2.5 μM, partial at 5 μM, but no reversal was seen at 10 μM. Several *E* isomers

TABLE 3. Antiproliferative activity of side chain analogs of clomiphene



Compound*†	R	R_1	R_2	IC_{50}‡ (μM) MCF-7	LY2	MDA-MB-231
38	H	H	H	> 10	> 10	ND§
39	H	OCH_3		2.1	3.5	ND
40	Cl	H	H	> 10	> 10	ND
41	Cl	H	$O(CH_2)_2N(C_3H_7)_2$	5.4	2.5	5.8
42	Cl	H	$O(CH_2)_2NH_2$	7.0	8.0	ND
43	Cl	H	$O(CH_2)_2NHCH_2CH_3$	0.9	5.0	> 10
44	Cl	H	$OCH_2CHCH_3N(CH_2CH_3)_2$	1.0	3.0	6.0
45	Cl	H	$O(CH_2)_3N(CH_2CH_3)_2$	0.97	4.5	ND
46	Cl	H	$O(CH_2)_4N(CH_2CH_3)_2$	0.64	0.6	7.8
47	Cl	H		0.6	2.0	6.6
48	Cl	H		0.2	1.0	7.4
49	Cl	H		3.0	3.0	> 10
50	Cl	H		0.1	1.4	> 10
51	Cl	OCH_3		0.1	0.9	ND
52	Cl	H		2.3	ND	ND
53	Cl	H		4.3	ND	ND
54	Cl	H		> 10	ND	ND
55	Cl	H	$(CH_2)_3N(CH_2CH_3)_2$	0.3	1.6	7.0
56	Cl	H	$S(CH_2)_2N(CH_2CH_3)_2$	0.4	1.0	5.9
57	Cl	H	$N(CH_2)_2N(CH_2CH_3)_2$	0.7	4.0	6.2
58	Tamoxifen			2.23	7.5	7.0
59	4-Hydroxytamoxifen			0.03	0.62	ND
15‖	Cl	H	$O(CH_2)_2N(CH_2CH_3)_2$	0.8	1.5	7.2

* Compounds are citrate salts except for **39**, **42**, **49** and **51** which are HCl salts.

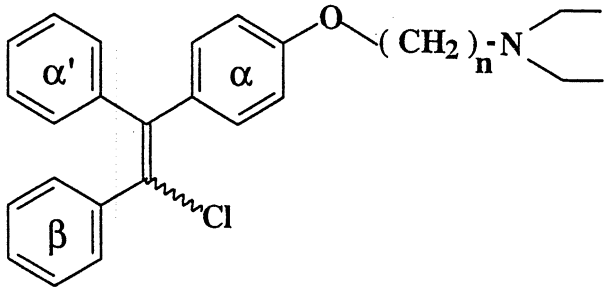
† Isomer configurations: all isomeric mixtures except for the configurations of **39** and **42** are unknown; **51** is an *E* isomer; **58** and **59** have *E* configurations, but *E*/*Z* priority rules enforce *Z* isomer designations.

‡ Most IC_{50} values ≤1 μM are means of at least two determinations.

§ ND = not determined.

‖ Clomiphene.

TABLE 4. Biological and biochemical activities of isomers with consecutive side chain extensions



		E isomers*			*Z* isomers†		
Compound	$(CH_2)_n$	Growth‡	pVETLUC§	RBA‖	Growth‡	pVETLUC§	RBA‖
15	2	2.2 ± 0.33	0.5 ± 0.02	1.6 ± 0.2	3.8	22	0.13
45	3	0.7 ± 0.1	0.2 ± 0.06	6.7 ± 1.4	NA¶	NA	NA
46	4	0.56 ± 0.1	0.1 ± 0.03	9.2 ± 1.2	4.2	2	0.24
60	5	1.4 ± 0.2	0.3 ± 0.04	1.9 ± 0.1	7.2	8	0.11
61	6	2.7 ± 0.5	0.6 ± 0.09	2.2 ± 0.9	4.6	9	0.17
62	7	1.8 ± 0.9	7.2 ± 1.5	2.9 ± 0.1	2.7	9	0.21
63	8	1.8 ± 0.7	1.2 ± 0.2	0.4 ± 0.03	3.4	7	0.07
64	9	1.9 ± 0.8	1.3 ± 0.2	0.22 ± 0.03	2.7	3	0.12
65	10	1.6 ± 0.7	1.8 ± 0.06	0.07 ± 0.003	3.1	18	0.07
66	11	2.7 ± 0.5	6.6 ± 1.3	0.04 ± 0.003	3.8	21	0.04
67	12	3.3 ± 0.4	12 ± 1.4	0.02 ± 0.0	5.4	14	0.02
tam	NA	NA	NA	NA	2.23 ± 0.3	0.7 ± 0.08	1.3 ± 0.4

* Means ± SEM, where *E* isomers were tested 3 times in each assay except that **15**, **46** and tam were tested in 8, 13, and 9 antiproliferation assays, respectively.
† *Z* isomers were tested once.
‡ MCF-7 antiproliferation IC_{50} (μM).
§ IC_{50} (μM) antagonizing expression of luciferase.
‖ Relative binding affinity for MCF-7 ER.
¶ NA = not available.

showed biphasic concentration–response curves similar to that of compound **22** (data not shown).

Correlations of RBA and IC_{50} Values

RBA values were determined for several compounds by competition with [^{3}H] estradiol for MCF-7 ER and compared with antiproliferative IC_{50} values for those analogs, as shown in Table 6. The RBA values are listed in descending order and correlate with an ascending order of the IC_{50} values. This type of pattern has been reported previously for MCF-7 cells [29–32]. Analyses of the concentration–response curves of the analogs in Table 6 showed all curves to be parallel (slope values were in a range of −25 to −43 and were not significantly different), indicating that the analogs bind to the same site in a non-cooperative manner. Compound **22** (4-hydroxyclomiphene) and **59** (4-hydroxytamoxifen) showed the highest RBA and the lowest IC_{50} values; however, compound **22** is a mixture of *E* and *Z* isomers. Therefore, to determine whether the elevated binding activity of **22** was due primarily to the *E* isomer, the isomers of compound **22** were purified by HPLC and the configurations were verified by NMR. The RBA values were 285 for the *E* isomer and 16 for the *Z* isomer (data not shown). Paradoxically, the compound with the lowest RBA

TABLE 5. Estradiol reversal of the antiproliferative effects of *E* and *Z* isomers

		IC_{50} (μM)			
		MCF-7		LY2	
Compound	Isomer	−E2	+E2*	−E2	+E2*
15	*E*	2.2	4.4	1.7	5.6
	Z	4.5	7.0	3.4	4.3
23	*E*	1.0	2.2	0.72	4.2
	Z	2.9	2.1	2.6	3.8
24	*E*	0.76	2.2	0.62	4.0
	Z	3.4	4.1	2.7	4.8
27	*E*	1.1	3.1	0.62	4.6
	Z	2.1	1.8	2.6	2.9
46	*E*	0.56	3.5	0.62	5.6
	Z	2.3	2.9	3.5	5.8
48	*E*	0.76	2.7	0.4	3.6
	Z	1.9	1.7	1.8	2.3
55	*E*	0.62	3.9	0.31	6.5
	Z	2.7	3.5	4.2	6.2
56	*E*	0.45	3.0	0.9	4.8
	Z	2.0	2.5	2.9	3.4
57	*E*	0.25	3.0	0.45	5.2
	Z	2.2	2.5	7.2	8.4
58 (Tam)	*Z*†	2.23	5.8	7.5	7.9

* IMEM supplemented with 0.1 μM estradiol.
† Tamoxifen is a *trans* structure, but *E*/*Z* priority rules enforce the *Z* designation.

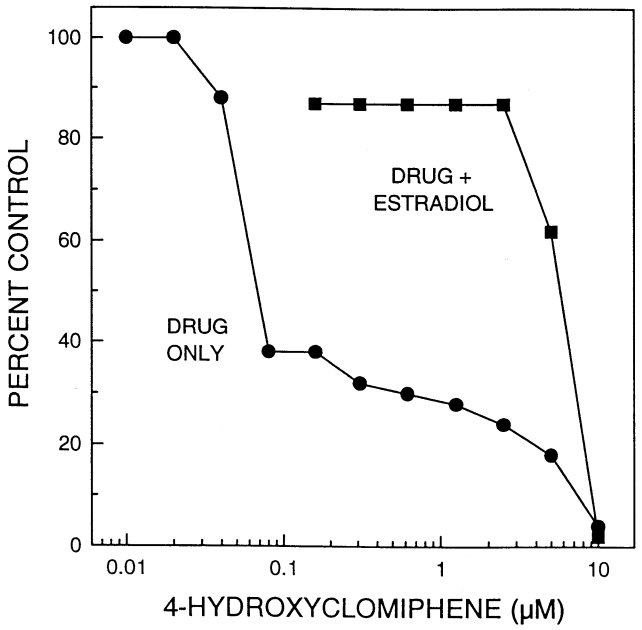


FIG. 1. Antiproliferative activity of 4-hydroxyclomiphene (22) and reversal by 0.1 μM estradiol. MCF-7 cells (1×10^4/well) were dispensed into 96-well microtiter plates, and after 24 and 96 hr the medium and drugs were renewed. After a total of 7 days of incubation, the cells were fixed and stained with sulforhodamine B, and the stain was extracted from the cells to determine absorbancies (492 nm) and percent control values.

and the highest IC_{50} (**16**) is also a 4-hydroxyclomiphene, but in **16** the hydroxy substitution is on the β ring (4′-hydroxyclomiphene) not the α′ ring as in **22**.

Antitumor Activity

Several analogs active in the antiproliferative assay were tested for activity toward MCF-7 tumor xenografts in nude mice. The results in Table 7 show that among the five compounds tested, **46** and **57** were greater than 3-fold more active than tamoxifen and greater than 10-fold more active than clomiphene.

TABLE 6. Correlation of ER binding affinities with antiproliferative effects in MCF-7 cells

Compound	Isomer	RBA*	IC_{50}† (μM)
22	*E* + *Z*	251	0.07
59	*E*	246	0.03
55	*E*	18	0.6
57	*E*	12	0.25
46	*E*	9	0.56
56	*E*	6	0.5
51	*E*	5	0.1
48	*E*	5	0.8
33	*E* + *Z*	3.3	0.7
24	*E*	2.6	0.8
15	*E*	1.6	2.2
27	*E*	1.6	1.1
23	*E*	1.3	1
58	*Z*	1.3	2
16	*E* + *Z*	<0.4	3

* Relative binding affinities for MCF-7 ER.
† Antiproliferative activity toward MCF-7 cells.

TABLE 7. Inhibition of MCF-7 tumor progression in mice by antiestrogens

Compound*	ED_{50} (mg/mouse)†
15 (clomiphene)	0.22
46	<0.02
55	0.085
56	0.29
57	<0.02
Tamoxifen	0.07

* Isomer configuration, *E*: **15**, **46**, **55** and **57**; *E* + *Z*: **56**.
† Tumor pieces were implanted into nude mice flanks by trocar; when tumor volumes reached 50–100 mm^3, treatment was begun by daily intraperitoneal administrations, 5 days/week for 6 weeks; N = 5–6 mice per treatment group.

DISCUSSION

In an antiproliferative screen of nearly 600 TPEs, we discovered clomiphene analogs with better activity *in vitro* and *in vivo* than clomiphene or tamoxifen. Of the 63 compounds presented here, 23 were active (IC_{50} values ≤1 μM) toward the growth of MCF-7 cells and 11 toward LY2 cells. TPE antiestrogens reported to have the most potent antiproliferative activity toward breast cancer cells contain side chains on the α ring [33], a feature common to all the compounds we identified as active. In addition, TPE antiestrogen-mediated growth antagonism is known to be reversible by estradiol [8, 34–36], and the growth antagonism by *E* isomers that we report here was reversed by estradiol supplementation as manifested by 3- to 20-fold increases in the IC_{50} values. Finally, it has been reported that TPE antiestrogens with the lowest antiproliferative IC_{50} values have the highest RBA values for the ER [8, 31, 37]. We found this correlation in a group of 15 compounds, mainly *E* isomers of clomiphene analogs.

Maximal antiproliferative and ER binding activity was shown with compound **22** (4-hydroxyclomiphene) against MCF-7 cells and with MCF-7 ER, respectively, (IC_{50}, 0.07 μM; RBA, 251). Also, several TPEs, including compound **22**, induced biphasic antiproliferation profiles with estradiol-reversible and -nonreversible components. Sutherland *et al.* [29] reported 4-hydroxyclomiphene to be the most active of several hydroxy TPEs against MCF-7, and that biphasic concentration-response profiles are helpful in defining different mechanisms of antiestrogen growth antagonism [29, 32]. The estradiol-reversible component suggests competition between the inhibitor and estradiol for binding to the ER [29–31], and the estradiol-nonreversible component may involve either inhibition of protein kinase C [38, 39] or calmodulin-dependent enzymes [29, 40], as the IC_{50} values of antiestrogens against these enzymes are in micromolar concentrations. It is apparent that the mechanism of growth antagonism by our most active analogs was compe-

tition for the ER, since the activities were reversed by estradiol. In addition, we tested 19 of the 23 active analogs against the ER negative cell line MDA-MB-231 and all were inactive; therefore, it seems unlikely that the antiproliferative effects toward MCF-7 and LY2 were due to nonspecific cytotoxic effects. Models have been proposed for the binding of estradiol and antiestrogens to the ER. The model proposed by Katzenellenbogen *et al.* [41] suggests two major sites, a ligand binding site and a ligand discriminating site. The model proposed by Jordan [15] accounts for both *E* and *Z* isomers, an antiestrogen region that accommodates binding of the alkyl ether side chain and a phenolic site responsible for low or high affinity binding, to which, it has been proposed, the 3-hydroxy group of estradiol binds. While both models have merit, the latter model helped put into perspective the markedly different RBAs of each 4-hydroxyclomiphene, **16** and **22**. Briefly, occupancy of the ER phenolic site by the 4-hydroxyl on the α ring of **22** (*E* isomer) would correlate with high-affinity binding; however, with **16** (*E* isomer) the 4-hydroxyl resides on the β ring, thus placing the 4-hydroxyl more distant from the phenolic site, and would correlate with low affinity binding. The binding affinities of the hydroxyclomiphene analogs **22** (RBA, 251) compared with **16** (RBA, <0.4) were similar to those of 4-hydroxytamoxifen (RBA, ≈ 250) compared with 4′-hydroxytamoxifen (OH on β ring and RBA ≈ 2.5) as shown by Ruenitz *et al.* [42] in competitive binding assays using rat uteri ER to compare hydroxytamoxifen structures. Taken together, these data show that for high affinity binding of both hydroxytamoxifen and hydroxyclomiphene, phenyl ring specificity is essential.

While none of the analogs were selective for LY2 cells (antiproliferative IC_{50} values $\geq$5-fold those of MCF-7), seven analogs (**22**, **43**, **48**, **50**, **51**, **55**, and **57**) were selective for MCF-7 cells, suggesting that the binding affinities of those analogs may be greater for the MCF-7 ER than for the LY2 ER. To examine this possibility, we compared RBA values for MCF-7 and LY2 ER, available for just 5 of the analogs (**48**, **50**, **51**, **55**, and **57**). Even though most of the RBA values were greater for the MCF-7 ER, none differed from LY2 by more than 20%, indicating that the selectivity of the analogs for MCF-7 was not related to ER binding differences. Mullick and Chambon [43] showed that the sequence of the coding region of the LY2 ER mRNA was the same as that of MCF-7, proving that LY2 ER is not a mutated form of the parent MCF-7 ER. Moreover, we estimated the K_d values for [^{3}H]estradiol to be the same (0.2 nM) with ER from both cell lines (data not shown). Bronzert *et al.* [18] also showed that the K_d value for [^{3}H]estradiol with MCF-7 ER is very similar to that of LY2. Thus, the resistance to tamoxifen and the decreased sensitivity to some TPEs shown here for LY2 do not seem to reside in the ER *per se*, but may be a consequence of the degree of estrogen independence in LY2 [43] and of LY2 cells containing just 43% of the number of ER sites in MCF-7 [18]. Still, the selectivity for MCF-7 versus LY2 remains unexplained but may be of interest for consideration of mechanisms of antiestrogen resistance.

Clomiphene can sustain more substitutions in the side chain than at the vinyl Cl position without concomitant reductions in antiproliferative activity toward MCF-7. For example, of the 17 clomiphene side chain analogs (**40–50**, **52–57**), 3 had about the same activity as clomiphene, 7 were more active, and 7 were less active than clomiphene. In contrast, with the 12 vinyl Cl analogs, just 3 (**27**, **33**, and **34**) were nearly as potent as clomiphene and the remaining 9 analogs were less active than clomiphene. Regarding 5 of the side chain analogs more active than clomiphene (**46**, **48**, **55**, **56**, and **57**) and 2 of the vinyl Cl analogs equipotent to clomiphene (**27** and **33**), we suggest that their activities are positively correlated to RBA values either greater than or the same as the RBA of clomiphene, respectively. However, more RBA values are needed before we would speculate whether ER binding potency is likely the reason additional side chain and vinyl Cl analogs were more or less active than clomiphene.

A number of vinyl Cl compounds with side chain heterocyclic groups showed the following antiproliferative potency ranking against MCF-7: 4-methyl piperazinyl (**50**) > piperidyl (**48**) > pyrrolidyl (**47**) > morpholinyl (**49**). Robertson *et al.* [44] reported similar rankings with tamoxifen side chain analogs and rat uterus ER competitive binding assays where 4-methyl piperazinyl > pyrrolidyl > piperidyl > morpholinyl and in rat antiuterotrophic assays 4-methyl piperazinyl and morpholinyl > pyrrolidyl > piperidyl. Antiproliferative data for MCF-7 cells with the above tamoxifen analogs could provide interesting structure–activity relationships compared with clomiphene side chain analogs. Side chain extensions with methylene groups showed that $(CH_2)_4$ in the side chain (**46**) (MDL 103,323) correlated with the best antiproliferative, antipVETLUC reporter expression and relative binding activities by an *E* isomer. The pVETLUC transcription assay is a sensitive indicator of competition between estradiol and inhibitor in intact cells, as is the relative binding assay in cell extracts. Thus, the potency of MDL 103,323 in both assays suggests that the antiproliferative activity of MDL 103,323 is directly related to its binding affinity for the ER with a subsequent down-regulation of transcription of genes having estrogen response elements. Interestingly, analogs of nafoxidine with $(CH_2)_3$ and $(CH_2)_4$ side chain extensions also showed maximal antiestrogen activity as antifertility agents in rats, as reported by Lednicer *et al.* [45].

Meanwhile, further studies with MDL 103,323 have been completed to evaluate its potential for treatment and chemoprevention of breast cancer. A study recently published from our laboratory showed that mammary carcinomas induced by *N*-methylnitrosourea were inhibited 90% in rats treated for 11 weeks with 0.1 mg MDL 103,323/kg/day, suggesting potent chemopreventive activity [46].

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