Halogenated Analogues of Tamoxifen: Synthesis, Receptor Assay, and Inhibition of MCF7 Cells

DAVID J. YANG*x, TIMOTHY TEWSON‡, WAYNE TANSEY*, LI-REN KUANG*, GREGG REGER§, ABDALLAH CHERIF§, KENNETH C. WRIGHT*, R. GENE MOULT*, ROY S. TILBURY*, KEVIN CHU*, E. EDMUND KIM*, AND SIDNEY WALLACE*

Received January 31, 1991, from the *Division of Diagnostic Imaging and the §Division of Medical Oncology, University of Texas M.D. Anderson Cancer Center, Box 57, 1515 Holcombe Boulevard, Houston, TX 77030, and the ‡Positron Diagnostic Research Center, University of Texas Health Science Center, Houston, TX 77030. Accepted for publication August 15, 1991.

Abstract □ This study was conducted to develop a ligand for imaging estrogen-receptor-positive breast tumors by positron emission tomography or single photon emission computed tomography. We synthesized fluoro and iodo analogues of tamoxifen, and these halogenated analogues produced greater affinity for binding to the receptor than tamoxifen. Values of the inhibition affinity constants were as follows: tamoxifen, 15 000 nM; fluoromethyl-*N,N*-diethyltamoxifen, 2500 nM for the *cis* isomer and 500 nM for the *trans* isomer; and iodomethyl-*N,N*-diethyltamoxifen, 1500 nM for the *cis* isomer and 1000 nM for the *trans* isomer. In studies of human MCF7 breast tumor cell growth, concentrations that inhibited tumor growth in 50% of the cases were as follows: tamoxifen, 11 μM; fluoromethyl-*N,N*-diethyltamoxifen, 4.5 and 11.8 μM for the *cis* and *trans* isomers, respectively; and iodomethyl-*N,N*-diethyltamoxifen, 2.4 and 6.3 μM for the *cis* and *trans* isomers, respectively. These studies suggest that both fluoro and iodo analogues of tamoxifen may be useful diagnostic compounds for predicting the response of estrogen-receptor-positive breast tumors to tamoxifen analogues used in chemotherapy.

Each year 150 000 American women are stricken by breast cancer, and nearly 44 000 women annually die of the disease. Endocrine therapy, one of the oldest nonsurgical methods for the treatment of breast cancer, is still considered standard for certain subsets of patients, typically postmenopausal women whose primary tumors have high levels of estrogen receptors.[1-3] Observations of significant changes in the binding of estrogen receptors by [^{18}F]estradiol in breast tumor patients were reported.[4]

Tamoxifen (1), a potent antiestrogen that binds to cytoplasmic estrogen receptors, has been widely used in this type of therapy. Compared with other hormonal treatments, tamoxifen has few side effects.[5,6] Also, tamoxifen–estrogen receptor complexes in the cell nucleus last longer (3 days) than estradiol–estrogen receptor complexes.[5] The purpose of this study was to develop agents for imaging estrogen receptors by positron emission tomography or single photon emission computed tomography. Such agents may predict the efficiency of tamoxifen therapy for breast tumors.

In previous reports, we described the synthesis and in vitro estrogen receptor assay of fluorotamoxifens with the fluorine atom placed on the ethyl chain.[7] We observed, however, that the tosyl (*p*-toluenesulfonyl) group is eliminated in the presence of the stronger base and that the formation of the butadiene by-product limits its in vivo use in imaging estrogen receptors. Increasing the ethyl chain by one carbon results in the synthesis of methyltamoxifen analogues, which are more stable in tosyl elimination.[7] Substitution of *N,N*-diethyl for the *N,N*-dimethyl portion of tamoxifen resulted in a fourfold increase in binding affinity.[8] Therefore, *N,N*-diethyl halogenated analogues were prepared for preliminary evaluation. In this paper, we report the synthesis, in vitro assay of binding to the estrogen receptor, and use in studies of human breast tumor MCF7 cell growth of hydroxymethylfluoromethyl- and iodomethyl-*N,N*-diethyltamoxifen (see structures).

Experimental Section

Materials—Estradiol and tamoxifen were obtained from Sigma Chemical Company (St. Louis, MO). Thin-layer chromatographic analysis was performed on Whatman K6F silica gel-packed plates (3 × 5 cm, 250 μm; Anaspec, MI). [^{3}H]Estradiol (specific activity, 158 Ci/mmol) for receptor binding was purchased from Amersham (Arlington Heights, IL). High-performance liquid chromatography (HPLC) was carried out on an LDC system, consisting of LDC ConstaMetric pumps, a Rheodyne injector, and a Spectra Physics model SP8450 variable UV–visible detector. ^{1}H NMR spectra were obtained from a GE 300-MHz instrument, and mass spectral data were obtained by direct probe analysis (Finnigan MAT INCOS-50) at The University of Texas Health Science Center (Houston, TX). Elemental analysis was performed by Galbraith Laboratories (Knoxville, TN).

Synthesis—*trans-1-[4-(2-Diethylaminoethoxy)phenyl]-1,2-diphenyl-5-tosyl-1-pentene (Z-Tosylmethyl-N,N-diethyltamoxifen; 3)*—*trans*-Hydroxymethyl-*N,N*-diethyltamoxifen (500 mg, 1.17 mmol; 2)[7] was dissolved in methylene chloride (20 mL), and the solution was cooled to 0 °C. Pyridine (0.66 mL) and tosyl chloride (266 mg, 1.40 mmol) were added. After 6 h, the reaction mixture was diluted with additional methylene chloride (20 mL) and washed with water, dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness to yield 420 mg of crude mixture. This crude mixture was chromatographed on a silica gel-packed column with ether:petroleum ether (1:1, 100 mL), followed by 10% triethylamine in ether:petroleum ether (1:1, 200 mL) as eluant to yield the purified *Z*-tosyl analogue of 3 (345 mg, 50%, oil): *m/z* 583 (40, M^+), 411 (20, $^+SO_3PhCH_3$); ^{1}H NMR δ 1.10 (t, J = 7.1 Hz, 6, CH_3CH_2N), 1.35 (s, 3, SO_3PhCH_3), 1.66 (pentet, J = 7.1 Hz, 2, $CH_2CH_2CH_2OSO_2PhCH_3$), 2.43 (t, J = 6.4 Hz, 2, OCH_2CH_2N), 2.57 (q, J = 7.1 Hz, 4, CH_3CH_2N), 2.77 (t, J = 6.4 Hz, 2, $CH_2CH_2CH_2OSO_2$), 3.84–3.92 (m, 4, OCH_2CH_2N and $CH_2CH_2CH_2OSO_2PhCH_3$), 6.54 (d, J = 6.8 Hz, 2, ArH 3,5 to OCH_2), 6.74 (d, J = 6.8 Hz, 2, ArH 2,6 to OCH_2), 7.00–7.33 (m, 12, ArH), 7.67 (d, J = 8.3 Hz, 2, ArH 2,6 to OSO_2).

trans-1-[4-(2-Diethylaminoethoxy)phenyl]-1,2-diphenyl-5-fluoro-1-pentene (Z-Fluoromethyl-N,N-diethyltamoxifen; 4)—The *Z*-tosyl analogue of 3 (120 mg, 0.2 mmol) was dissolved in tetrahydrofuran (1 mL), and tetrabutylammonium fluoride (0.5 mL, 1 M in tetrahydrofuran) was added. The reaction was warmed to 50 °C. After 30 min, the reaction mixture was cooled and chromatographed on a silica gel-packed column, which was eluted with 10% triethylamine in

Compound	R	X
1 (Tamoxifen)	CH_3	H
2	C_2H_5	CH_2OH
3	C_2H_5	CH_2OTs
4	C_2H_5	CH_2F
5	C_2H_5	CH_2I

0022-3549/92/0700-0622$02.50/0

Table I—^{13}C (50-MHz) and ^{1}H (200-MHz) NMR Assignments for Fluoromethyl-*N,N*-diethyltamoxifen (in $CDCl_3$)

Atom	^{1}H Signal (± 0.02), ppm		No. of H	^{1}H Coupling ($J_{H,H}$, Hz)		No. of C	^{13}C Signal, ppm ($J_{H,H}$, Hz)	
	cis	*trans*	*cis/trans*	*cis*	*trans*	*cis/trans*	*cis*	*trans*
Ar	7.23	7.25	10H	m	m	6C	130–187	130–157
						10C	126–132	126–132
a	7.10	6.79	2H	m	d (6.8)	1C	114	113.5
b	7.00	6.56	2H	m	d (6.8)	1C	114.2	113.5
3	4.38	4.42	2H	dt (47.3, 6.1)	dt (47.3, 6.1)	1C	83.5 (d, 165)	85.2 (d, 165)
5	3.99	3.91	2H	t (6.37)	t (6.4)	1C	66.6	66.3
4	2.80	2.79	2H	t (6.37)	t (6.4)	1C	51.9	51.7
6	2.57	2.56	4H	m	m	2C	47.9	47.8
1	2.76	2.55	2H	m	m	1C	31.5 (d, 5.5)	31.6 (d, 5.5)
2	1.79	1.80	2H	m	m	1C	29.9 (d, 19.5)	29.8 (d, 44.3)
7	1.01	1.03	6H	t (7.2)	t (7.2)	2C	11.8	11.8

ether:petroleum ether (1:1) to yield 50 mg (58%, oil) of the purified *Z*-isomer fluoro product (4). *Anal.*—($C_{29}H_{34}NOF$) C,H,N. Calc.: C, 80.71; H, 7.94; N, 3.25; Found: C, 80.39, H, 8.02, N, 3.23 [*m/z* 431 (50, M^+); ^{13}C and ^{1}H NMR chemical shifts of 4 are presented in Table I].

1-[4-(2-Diethylaminoethoxy)phenyl]-1,2-diphenyl-5-iodo-1-pentene (Iodomethyl-N,N-diethyltamoxifen; 5)—The *E*- or *Z*-tosyl hydroxymethyl analogue of tamoxifen (3; 117 mg, 0.2 mmol) was dissolved in acetone (15 mL). Sodium iodide (150 mg, 1.0 mmol) was added, and the reaction was refluxed for 6 h. The mixture was evaporated to dryness, reconstituted in methylene chloride (30 mL), and washed with water (3 × 10 mL). The methylene chloride layer was separated, dried over anhydrous magnesium sulfate, filtered, and evaporated. The crude mixture was chromatographed on a silica gel-packed column eluted with 10% triethylamine in ether:petroleum ether (1:1) to yield the *E* isomer in a yield of 75 mg (70%) and the *Z* isomer in a yield of 54 mg (50%; retardation factor, 0.65 with 10% triethylamine in ether:petroleum ether, 1:1). *Anal.* (for the *E* isomer)—($C_{29}H_{34}NOI \cdot 1/3H_2O$) C,H,N. Calc.: C, 63.85; H, 6.40; N, 2.56; Found: C, 62.87; H, 6.35; N, 2.38. *Anal.* (for the *Z* isomer)—($C_{29}H_{34}NOI$) C,H,N. Calc.: C, 64.56; H, 6.35; N, 2.60; Found: C, 64.41; H, 6.47; N, 2.53. ^{13}C and ^{1}H NMR assignments of 5 are presented in Table II.

In Vitro Assay of Estrogen Receptor—The affinity of the test compounds for binding to the estrogen receptor was determined with a modification of the procedure reported by others.[9,10] Briefly, the uterus (30 g) obtained from a domestic swine (30 kg) was homogenized in Tris [tris (hydroxymethyl) aminomethane] buffer (10 mM, pH 7.4, 80 mL), which contained EDTA (1.5 mM) and sodium azide (3 mM). The homogenate was centrifuged at 1 000 000 × *g* for 1 h at 4 °C. Uterus cytosol was then pretreated with dextran-coated charcoal as described previously.[9] To investigate the nature of estradiol interaction with the estrogen receptor site, a saturation curve was obtained for [^{3}H]estradiol (10^{-5}–10^{-10} M) in the presence or absence of excess estradiol (10^{-5}M). Uterus cytosol was incubated at 4 °C for 2 h with [^{3}H]estradiol (5 nM/tube) and competitor (10^{-3}–10^{-8} M) or with estradiol (10^{-5} M; nonspecific). The concentrations of test compounds that decreased specific radioligand binding by 50% (IC_{50}) were measured. Protein concentrations were determined according to the method of Lowry et al.[11]

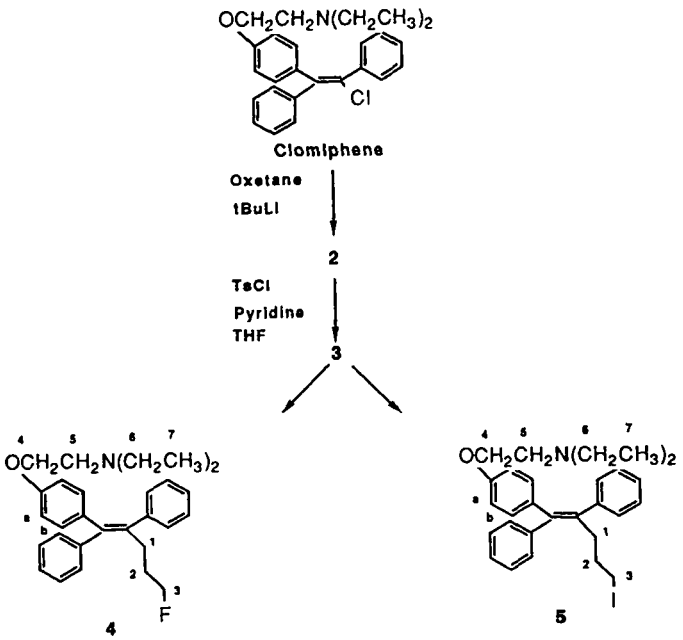
MCF7 Cell Growth Assay—The human breast tumor MCF7 cell line was cultured in modified Eagle medium in a 5% CO_2 atmosphere with 10% fetal calf serum. The medium was supplemented with 1 mM sodium pyruvate and 100 μM of nonessential amino acids. The cell line was screened routinely for mycoplasma contamination by using the GenProbe kit (Fisher Scientific, Houston, TX). Cells were trypsinized and plated at a density of 5000 cells/well in 96-well microtiter plates and allowed to attach and recover for 24 h. The medium was removed by aspiration and replaced with filter-sterilized drug (concentration, 10^{-4}–10^{-8} M) in medium. The cells were incubated for 72 h and then stained by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium dye assay of Mosmann,[12] except that after the medium was removed, the blue formazan product was solubilized in 50 μL/well of dimethyl sulfoxide. Plates were shaken for 1 min and read on a Dynatech MR600 microplate reader within 1 h at a transmission wavelength of 570 nm and a reference wavelength of 630 nm.

Results and Discussion

Chemistry—Fluoromethyltamoxifen (4) and iodomethyl-*N,N*-diethyltamoxifen (5) were prepared from the corresponding tosylmethyl-*N,N*-diethyltamoxifen directly via a reaction with tetrabutylammonium fluoride or sodium iodide. The synthesis of 4 and 5 has been simplified to a three-step procedure (Scheme I), which is also suitable for radiosynthesis of tamoxifen analogues with high specific activity.[13] For instance, [^{18}F]tamoxifen analogue can be prepared by reacting [^{18}F]tetrabutylammonium fluoride with the tosyl analogue of tamoxifen, and the ^{123}I-labeled analogue can be prepared by reacting $Na^{123}I$ with the tosyl analogue of tamoxifen. Both *E* and *Z* isomers can be isolated by passing the reaction mixture through a silica gel-packed column, and

Table II—^{13}C (50-MHz) and ^{1}H (200-MHz) NMR Assignments for Iodomethyl-*N,N*-diethyltamoxifen (in $CDCl_3$)

Atom	^{1}H Signal (± 0.02), ppm		No. of H	^{1}H Coupling ($J_{H,H}$, Hz)		No. of C	^{13}C Signal, ppm	
	cis	*trans*	*cis/trans*	*cis*	*trans*	*cis/trans*	*cis*	*trans*
Ar	7.20	7.40	10H	m	m	6C	135–157	135–157
						10C	126–131	126–131
a	7.10	6.76	2H	m	d (6.8)	1C	114.3	113.37
b	7.00	6.54	2H	m	d (6.8)	1C	114.3	113.37
3	2.88	2.78	2H	t (6.4)	t (6.4)	1C	6.19	6.38
5	4.06	3.90	2H	t (6.4)	t (6.4)	1C	66.64	66.16
4	3.04	3.02	2H	t (7.0)	t (7.1)	1C	51.85	51.59
6	2.60	2.60	1H	m	m	2C	47.89	47.77
1	2.60	2.60	2H	m	m	1C	37.06	37.05
2	1.86	1.86	2H	Pent (7.4)	Pent (7.4)	1C	32.95	32.92
7	1.02	1.02	6H	t (7.1)	t (7.1)	2C	11.95	11.77



Scheme I—Synthetic scheme of fluoro- and iodomethyl-*N,N*-diethyltamoxifen.

they can be eluted with 10% triethylamine in ether:petroleum ether (1:1). ^{1}H NMR chemical shift signals for *E* and *Z* isomers of tamoxifen-based analogues were assigned on the basis of published information.[14] With two-dimensional NMR techniques to analyze our tamoxifen analogues, the chemical shifts for fluorine atom (attached to carbon-3) were 4.40 ppm (dt, J = 47.3, 6.1 Hz, ^{1}H NMR) and 114 ppm (^{13}C NMR; Table I). The chemical shifts for the iodine atom (attached to carbon-3) were 2.78 ppm (t, J = 6.4 Hz, ^{1}H NMR) and 6.38 ppm (^{13}C NMR; Table II). As a result, the chemical shifts of fluorine and iodine atoms attached to carbon-3 observed by ^{1}H NMR or ^{13}C NMR are significantly different.

In Vitro Assay of Binding to Estrogen Receptor—For [^{3}H]estradiol binding in pig uterus (Figure 1), Scatchard analysis indicated a single class of binding sites with a mean binding affinity constant of 5 nM (n = 9) and a mean receptor density of 376 fmol/mg of protein (Figure 2). The protein concentration used was 1 mg/mL of cytosol. Hill analysis (coefficient, 0.982) indicated that estradiol has competitive reversible binding. The relative binding affinities of ligands to estradiol were determined (Table III). To explore the relationship between lipophilic character and affinity to bind estrogen receptor, we compared the halogenated tamoxifens to hydroxymethyl-*N,N*-diethyltamoxifen. In general, the binding of the *Z* isomer to the estrogen receptor is greater than that of the *E* isomer. The affinity of the tamoxifen analogues for binding to the receptor was, in decreasing order: fluoro- > iodo- > hydroxy- > tamoxifen. The lipophilicity of these analogues seems to be weakly correlated with their binding affinity. (*Z*)-Fluoromethyl-*N,N*-diethyltamoxifen binds to estrogen receptor 30-fold more strongly than does tamoxifen. (*Z*)-iodomethyl-*N,N*-diethyltamoxifen binds to estrogen receptor 15 times greater than does tamoxifen and two times less than does the fluoro analogue. The data suggest that both *Z* isomers of fluoro- and iodotamoxifen analogues are potentially useful for imaging estrogen receptors by positron emission tomography or single photon emission computed tomography.

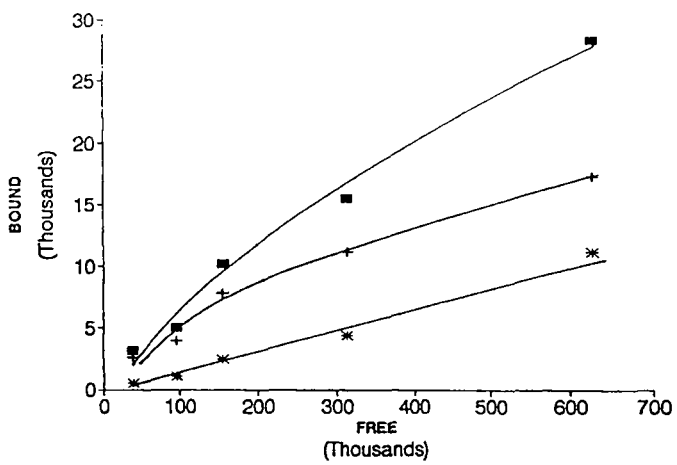


Figure 1—Binding to estrogen receptors. Key: (■) total; (+) specific; (*) nonspecific.

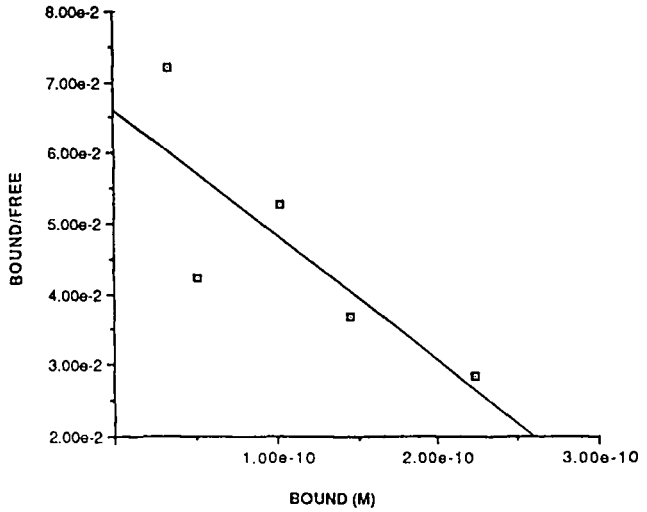


Figure 2—Scatchard analysis of in vitro saturation experiment and estrogen receptor assay.

MCF7 Cell Growth Assay—The concentration of test compounds required to inhibit 50% (IC_{50}) of MCF7 cell growth was determined (Table III). The relative potency of tamoxifen analogues was in decreasing order: iodo- > fluoro- > tamoxifen > hydroxy-. Unlike binding to the estrogen receptor, the lipophilic character of the analogue

Table III—Effects of Tamoxifen Analogues on Binding to Estrogen Receptor in Pig Uterus and on Human Breast Tumor Cell Growth

Compound	IC_{50}, μM[a]	RBA[b]	IC_{50}, μM[c]	RP[d]
(*Z*)-**1**	30 ± 1.2	1	11.0 ± 0.21	100
(*E*)-**2**	10 ± 0.7	3	16.7 ± 0.04	66
(*Z*)-**2**	7 ± 0.3	4	22.0 ± 0.12	50
(*E*)-**4**	5 ± 0.3	6	4.5 ± 0.04	224
(*Z*)-**4**	1 ± 0.4	30	11.8 ± 0.40	93
(*E*)-**5**	3 ± 0.2	10	2.4 ± 0.02	466
(*Z*)-**5**	2 ± 0.4	15	6.3 ± 0.03	175

[a] IC_{50} indicates the concentration of test compounds that decreased specific radioligand binding by 50%. Data represent average of three experiments; for each experiment, data were reproduced in triplicate. [b] Relative binding affinity (RBA) is the ratio of the concentration of tamoxifen to that of competitor (×100%) required to decrease the amount of bound [^{3}H]estradiol by 50%. [c] IC_{50} indicates the concentrations of test compounds required to inhibit 50% of MCF7 cell growth; data represent average of three studies of each compound. [d] Relative potency (RP) is the ratio of the IC_{50} of tamoxifen to that of competitor (×100%).

seemed to have an important role in cell growth inhibition. The iodo compound produced the greatest cytostatic effect (in addition, its *E* isomer was more potent than its *Z* isomer). It has been reported that, among nonhalogenated tamoxifen analogues, a partial *E* isomer can convert to the more active *Z* isomer during cell culture experiments.[15,16] Our data indicate that the *E* isomer is more lethal than the *Z* isomer. This lethal effect could be due to the intramolecular hydrogen bonding provided by the halogenated *cis* isomer, which may help it chelate to the phospholipids in the tumor cell membranes. This chelation process might result in a change in membrane permeability and cause cell death. Also, the electrical inductive effect produced by halogens may fit the estrogen receptor model well so that halogenated tamoxifen analogues have a greater affinity for the estrogen receptor than tamoxifen. The iodo analogue showed a fivefold greater potency than that of tamoxifen. The finding that both fluoro and iodo analogues were more potent than tamoxifen suggests that these analogues may be useful in estrogen-receptor-positive breast tumor therapy.

In summary, increasing the side chain of tamoxifen by one carbon resulted in the synthesis of the methyltamoxifen analogue, which is more stable toward elimination. The synthetic procedure we report here is also suitable for producing high specific radioactivity. Both fluoro and iodomethyl analogues of tamoxifen produced greater binding to the estrogen receptor and better breast tumor cell inhibition than tamoxifen. These findings encourage the further testing of these halogenated analogues of tamoxifen for in vivo studies.

References and Notes

1. Wittliff, J. L. *Cancer Res.* 1984, *53*, 630–643.
2. McGuire, W. L.; Horowitz, K. B.; Pearson, O. H.; Segaloff, A. *Cancer* 1977, *39*, 2934–2947.
3. Fernandez, M. D.; Burn, J. I.; Sauven, P. D.; Parmar, G.; White, J. O.; Myatt, L. *Eur. J. Cancer Clin. Oncol.* 1984, *20*, 41–46.
4. Mintun, M. A.; Welch, M. J.; Siegel, B. A.; Mathias, C. J.; Brodack, J. W.; McGuire, A. H.; Katzenellenbogen, J. A. *Radiology* 1988, *169*, 45–48.
5. Kallio, S.; Kangas, L.; Blanco, G.; Johansson, R.; Karjalainen, A.; Perila, M.; Pippo, I.; Sundquist, M.; Sodervall, M.; Toivola, R. *Cancer Chemother. Pharmacol.* 1986, *17*, 103–108.
6. Kangas, L.; Nieminen, A.-L.; Blanco, G.; Gonroos, S.; Kallio, S.; Karjalainen, A.; Perila, M.; Sodervall, M.; Toivola, R. *Cancer Chemother. Pharmacol.* 1986, *17*, 109–113.
7. Yang, D. J.; Wallace, S.; Tansey, W.; Wright, K. C.; Kuang, L.-R.; Tilbury, R. S.; Diego, I.; Lim, J. L.; Emran, A. M.; Kim, E. E. *J. Pharm. Res.* 1991, *8*, 174–177.
8. Foster, A. B.; McCague, R.; Seago, A.; Leclercq, G.; Stoessel, S.; Roy, F. *Anticancer Drug Design* 1986, *1*, 245–257.
9. Fishman, J. H. *Biochem. Biophys. Res. Commun.* 1983, *110*, 713–718.
10. McCague, R.; Leclercq, G.; Jordan, V. C. *J. Med. Chem.* 1988, *31*, 1285–1290.
11. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* 1951, *193*, 265–275.
12. Mosmann, T. *J. Immunol. Methods* 1983, *65*, 55–63.
13. Hamacher, K.; Coenen, H. H.; Stöcklin, G. *J. Nucl. Med.* 1986, *27*, 235–238.
14. Foster, A. B.; Jarman, M.; Leung, O.-T.; McCague, R.; Leclercq, G.; Devleeschouwer, N. *J. Med. Chem.* 1985, *28*, 1491–1497.
15. McCague, R.; Leclercq, G. *J. Med. Chem.* 1987, *30*, 1761–1767.
16. Katzenellenbogen, B. S.; Norman, M. J.; Eckert, R. L.; Peltz, S. W.; Mangel, W. F. *Cancer Res.* 1984, *44*, 112–119.

Acknowledgments

This work was supported in part by a seed grant from the Radiological Society of North America, the John S. Dunn Foundation, the George and Cleo Cook Fund, and the Cancer Fighters of Houston. We thank Dianne Perez–Onuogu and LaDonna McClain for helping in the preparation of the manuscript. Presented in part at the 200th Annual Meeting of the American Chemical Society, August 26–31, 1990 (Washington, DC).