Synthesis and *in Vitro* Receptor Binding Studies of Fluorotamoxifen Analogues[1]

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Received June 29, 1990; accepted August 9, 1990

We describe the synthesis of new fluorotamoxifen analogues with the fluorine atom positioned on the end of the aliphatic chain of tamoxifen. The binding of fluorotamoxifens to cytosol estrogen receptors of rat uteri was determined with [^{3}H]estradiol (5 n*M*). The fluorotamoxifens had similar or superior binding affinities compared with tamoxifen. The IC_{50} value was as follows: tamoxifen, 5×10^{-7} M; fluorotamoxifen (VII), 5×10^{-7} *M*; *N,N*-diethylfluorotamoxifen (IV)—*cis*, 1×10^{-6} *M*, and *trans*, 2×10^{-7} *M*; and (*cis*) fluoromethyl-*N,N*-diethyltamoxifen (VI) 1×10^{-7} *M*. Therefore, the fluorinated tamoxifens have potential use in imaging estrogen receptors by PET.

KEY WORDS: estrogen; receptor; fluorotamoxifen; synthesis.

INTRODUCTION

Tamoxifen (I), a potent nonsteroidal antiestrogen, has been widely used in the treatment of human breast tumors and has few side effects when compared with other hormonal treatments. Tamoxifen is cytostatic and exerts competitive inhibitory activity at the receptor level with estrogen. It binds to cytoplasmic estrogen receptors and is translocated to cell nuclei, where cell proliferation is prevented (1–3). The primary purpose of this study was to develop potential PET agents for imaging estrogen receptors. Such agents may predict the efficiency of tamoxifen therapy for breast tumors. Significant changes in the binding of estrogen receptors in breast tumor were reported with the use of [^{18}F]fluoroestradiol using positron emission tomography (PET) (4). Aliphatic fluorination of tamoxifen has been our choice based on the relative ease of the use of such route and expected high specific radioactivity (5). The addition of fluorine to the aliphatic side chain, rather than to the aromatic portion of the molecule, preserves the major portion of the molecule for binding with a minimum of alteration. Also, substitution of *N,N*-diethyl for the *N,N*-dimethyl portion of tamoxifen has been demonstrated to increase binding to the estrogen receptor by four times (6). Therefore, the *N,N*-dimethyl (VII) and the *N,N*-diethyl (IV and VI) fluoro analogues of tamoxifen were prepared for preliminary evaluation.

EXPERIMENTAL

Materials

Clomiphene, estradiol, and tamoxifen were obtained from Sigma Chemical Company (St. Louis, MO). Flash chromatography was used by the procedure of Still *et al.* (7) and silica gel Sep-Paks from Waters Associates (Milford, MA) were used for purifications. Thin-layer chromatographic (TLC) analysis was performed on Whatman K6F silica gel-packed plates (250 μm) (Anspec, MI). [^{3}H]Estradiol (sp act, 140–170 Ci/mmol) for receptor binding was purchased from Amersham (Arlington Heights, IL). High-pressure liquid chromatography (HPLC) was carried out on an LDC system, consisting of two LDC ConstaMetric pumps, a Rheodyne injector, and a Spectra Physics Model SP8450 variable UV/Vis detector.

Melting points were determined on a Meltemp melting-point apparatus and are uncorrected. ^{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. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

Synthesis

trans-*Fluorotamoxifen (VII)*

Hydroxytamoxifen (V) (8) (330 mg, 0.85 mmol) was dissolved in methylene chloride (20 ml), cooled to −40°C, and then treated with triethylamine (200 μl) added. Diethylaminosulfur trifluoride (250 μl, 1.89 mmol) was added and the reaction mixture was stirred for 1 hr at −40°C according to our previous published method (9). The reaction mixture was then washed with water and the methylene chloride layer evaporated to dryness. The reaction mixture was chromatographed on a silica gel column using 1:1:0.1 hexane/ethylacetate/triethylamine as eluant to yield 145 mg (43.7%) of VII: R_f 0.40 (1:1:0.1 ether/petroleum ether/triethylamine); ^{1}H-NMR ($CDCl_3$) δ 2.29 (S, 6, NMe_2) 2.66 (t, J = 5.6 Hz, 2, OCH_2CH_2N), 2.87 (dt, J = 21.2 Hz, 6.3 Hz, 2, CH_2CH_2F), 3.93 (t, J = 5.5 Hz, 2, OCH_2CH_2N), 4.34 (dt, J = 47.2 Hz, 6.3 Hz, 2, CH_2F), 6.56 (d, J = 8.5 Hz, 2, ArH 3, 5 to OCH_2), 6.77 (d, J = 8.3 Hz, 2, ArH 2, 6 to OCH_2), 7.12–7.35 (m, 10, ArH); *m/z* 389 (12, M^+), 342 (30, $^+CH_2$–CH_2–F).

N,N-*Diethylhydroxytamoxifen (II)*

Clomiphene (6.06 g, 14.9 mmol) was dissolved in tetrahydrofuran (100 ml) and cooled to −40°C. t-Butyl lithium (1 *M* in pentane, 24 mmol) was added slowly. After 5 min, ethylene oxide (14.6 ml, 290 mmol) was added, and the reaction mixture was stirred for 6 hr, poured into water, and extracted with ether. The ether layer was evaporated and

[1] Presented in part at the 4th Annual Meeting of the American Association of Pharmaceutical Scientists, October 22–26, 1989, in Atlanta, Georgia.

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0724-8741/91/0200-0174$06.50/0

chromatographed on a silica gel column using 1:1:0.1 ether/petroleum ether/triethylamine as eluant to yield *trans* product (1.96 g, 27.1%, oil) and *cis* product (1.56 g, 21.5%, oil): values for aliphatic protons are presented in Table II.

N,N-*Diethyl*-O-*tosyltamoxifen (VIII)*

cis or *trans N,N*-diethylhydroxytamoxifen (II) (110 mg, 0.27 mmol) was dissolved in methylene chloride (2 ml) and cooled to 0°C. Pyridine (150 µl) and tosyl chloride (55 mg, 0.27 mmol) were added. After 2 hr, the reaction mixture was diluted with methylene chloride and washed with water. The methylene chloride layer was evaporated and chromatographed on a ^{18}C column using 85:15:1 acetonitrile/water/triethylamine as eluant to yield *cis* (51 mg, 34%, oil) or *trans* tosyl analogue (30 mg, 20% oil): *m/z* 569 (60, M^+), 397 (20, $^+OSO_2PhCH_3$). Values for alphatic protons are presented in Table II.

N,N-*Diethylfluorotamoxifen (IV)*

N,N-Diethyl tosyl analogue of tamoxifen (VIII) (40 mg, 0.07 mmol) was dissolved in tetrahydrofuran (200 µl) and then treated with tetrabutylammonium fluoride (170 µl, 1 *M* in tetrahydrofuran). After 15 min, two spots were visualized by silica gel TLC (4:1 chloroform/methanol). Both products were isolated from a silica gel Sep-Pak by elution with ether/petroleum ether/triethylamine (1:1:0.1). One product isolated was the title compound (IV) (11 mg, 40%) and the other was a butadiene derivative (30%, oil). *trans* IV ^{1}H-NMR ($CDCl_3$) δ 1.02 (t, *J* = 7.3 Hz, 6, CH_3CH_2N), 2.57 (q, *J* = 7.1 Hz, 4 CH_3CH_2N), 2.78 (t, *J* = 6.3 Hz, 2, OCH_2CH_2N), 2.91 (dt, *J* = 21.5 Hz, 6.3 Hz, 2, CH_2CH_2F), 3.90 (t, *J* = 6.2 Hz, 2, OCH_2CH_2N), 4.33 (dt, *J* = 47.4 Hz, 6.3 Hz, 2, CH_2CH_2F), 6.56 (d, *J* = 8.5 Hz, 2, ArH 3, 5 to OCH_2), 6.75 (d, *J* = 8.7 Hz, 2, ArH 2, 6 to OCH_2), 7.12–7.37 (m, 10, ArH); *m/z* 417 (50, M^+). *Anal.* ($C_{28}H_{32}NOF \cdot 1/3\ H_2O$) C,H,N. *Calc.* C: 79.40, H: 7.70, N: 3, 31. *Found.* C: 79.71, H: 7.61, N: 3.36. *cis* IV ^{1}H-NMR ($CDCl_3$) δ 1.08 (t, *J* = 7.1 Hz, 6, Ch_3CH_2N), 2.64 (q, *J* = 7.3 Hz, 4, CH_3CH_2N), 2.89–2.96 (m, 4, OCH_2CH_2N and CH_2CH_2F), 4.06 (t, *J* = 6.4 Hz, 2, OCH_2CH_2N), 4.35 (dt, *J* = 47.1 Hz, 6.4 Hz, 2, CH_2CH_2F), 6.89–7.26 (m, 14, ArH); *m/z* 417 (70, M^+). m.p. 55–57°C. *Anal.* ($C_{28}H_{32}NOF \cdot 0.5\ H_2O$) C,H,N. *Calc.* C: 78.84, H: 7.80, N: 3.28. *Found.* C: 78.71, H: 7.48, N: 3.20. Butadiene derivative ^{1}H-NMR ($CDCl_3$) δ 1.08 (t, *J* = 7.0 Hz, 6, CH_3CH_2N), 2.65 (q, *J* = 7.0 Hz, 4, CH_3CH_2N), 2.90 (t, *J* = 6.0 Hz, 2, OCH_2CH_2N), 4.08 (t, *J* = 6.0 Hz, 2, OCH_2CH_2N), 4.94 (d, *J* = 17.2 Hz, 1, $CH=CH_2$), 5.17 (d, *J* = 10.9 Hz, 1, $CH=CH_2$), 6.78–7.26 (m, 14, ArH and $CH=CH_2$). *m/z* 397 (60, M^+). *Anal.* ($C_{28}H_{31}NO \cdot 1.5\ H_2O$) C,H,N. *Calc.* C: 79.21, H: 8.06, N: 3.30. *Found.* C: 79.76, H: 7.56, N: 3.09.

Hydroxymethyl-N,N-*diethyltamoxifen (III)*

Clomiphene (3.8 g, 9.3 mmol) was dissolved in tetrahydrofuran (50 ml), cooled to −40°C, and then treated with t-butyl lithium (1 *M* in pentane, 20 mmol). After 10 min, trimethylene oxide (6 ml, 93 mmol) was added, and the mixture stirred for 16 hr at room temperature and then poured into water. The product was extracted with ether and chromatographed on a silica gel column using 1:1:0.1 ether/petroleum ether/triethylamine as eluant to yield purified *trans* product (1 g, 25%), m.p. 93–95°C, and *cis* product (1.0 g, 25%), m.p. 85–87°C. *Anal.* ($C_{29}H_{35}NO_2$) C,H,N. *Calc.* C: 81.08, H: 8.21, N: 3.26. *Found.* C: 80.56, H: 7.94, N: 3.32. Values for aliphatic protons are presented in Table II.

cis-*Tosylmethyl*-N,N-*diethyltamoxifen (IX)*

(*cis*)Hydroxymethyl-*N,N*-diethyl tamoxifen (500 mg, 1.17 mmol) (III) was dissolved in methylene chloride (20 ml), and the solution cooled to 0°C. Pyridine (0.66 ml) and tosyl chloride (266 mg, 1.40 mmol) were added. After 4 hr, the reaction mixture was diluted with additional methylene chloride (20 ml) and washed with water, dried over magnesium sulfate, filtered, and evaporated to yield 476 mg. The crude mixture was chromatographed on a ^{18}C reverse-phase column using 85:15:1 acetonitrile/water/triethylamine as eluant to yield the purified *cis* tosyl analogue of IX (200 mg, 29%, oil), R_f 0.35 (silica gel plates; ether/petroleum ether/triethylamine, 1:1:0.1), *m/z* 583 (10, M^+). Values for aliphatic protons are presented in Table II.

cis-*Fluoromethyl*-N,N-*diethyltamoxifen (VI)*

The *cis* tosyl analogue of IX (117 mg, 0.2 mmol) was dissolved in tetrahydrofuran (400 µl) according to our reported procedure (9). Tetrabutylammonium fluoride (485 µl, 1 *M* in tetrahydrofuran) was added, and the reaction was warmed to 50°C. After 30 min, the reaction was completed. The product was chromatographed on a silica gel column, which was eluted with 1:1:0.1 ether/petroleum ether/triethylamine to yield 52 mg (60%, oil) of purified *cis* fluoro product (VI), R_f 0.80 (silica gel plates: ether/petroleum ether/

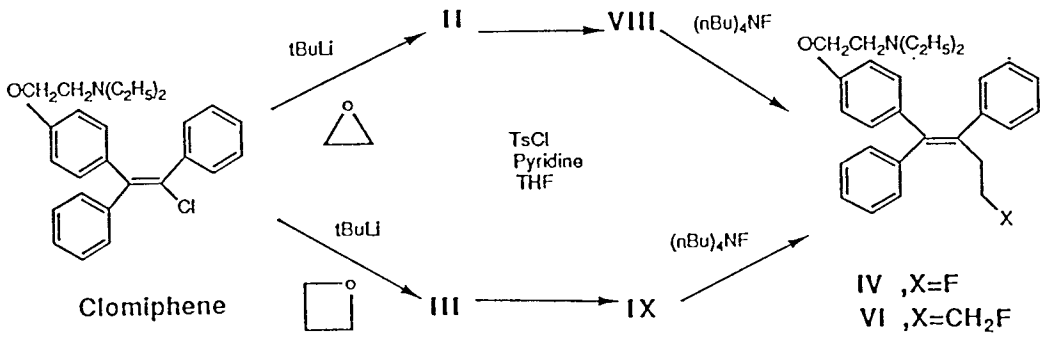
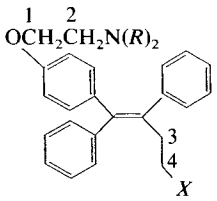


Fig. 1. Synthesis of tamoxifen derivatives.

Table I. Structures and Relative Binding Affinities of Tamoxifen Derivatives



Compound	*R*	*X*	RBA[a]	IC_{50} (*M*)	K_i (n*M*)
I (Tamoxifen)	CH_3	H	100	5×10^{-7}	333
II	C_2H_5	OH			
III	C_2H_5	CH_2OH			
IV					
(*cis*)	C_2H_5	F	50	1×10^{-6}	667
(*trans*)			250	2×10^{-7}	133
V	CH_3	OH			
VI	C_2H_5	CH_2F	500	1×10^{-7}	67
VII	CH_3	F	100	5×10^{-7}	333
VIII	C_2H_5	O-tosyl			
IX	C_2H_5	CH_2O-tosyl			

[a] The relative binding affinity (RBA) for the rat uteri estrogen receptor is the ratio between the concentration of unlabeled tamoxifen and the competitor (×100%) required to decrease the amount of bound [^{3}H]estradiol by 50%. Incubation was done at 4°C. The data were reproduced in triplicate.

triethylamine, 1:1:01). *cis* of VI ^{1}H-NMR δ 1.01 (t, J = 7.1 Hz, 6, CH_3CH_2N), 1.75 (d Pent, J = 22.0 Hz, 6.2 Hz, 2, $CH_2CH_2CH_2F$), 2.50–2.61 (m, 6, OCH_2CH_2N and CH_3CH_2N), 2.81 (t, J = 6.3 Hz, 2, $CH_2CH_2CH_2F$), 4.24 (dt, J = 47.3 Hz, 6.1 Hz, 2, $CH_2CH_2CH_2F$), 6.79–7.17 (m, 14, ArH); *m/z* 431 (40, M^+). *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: 3.13.

In Vitro *Estrogen Receptor Assay*

The affinity of the test compounds to bind the estrogen receptor was determined with modification of the reported procedure (10, 11). Briefly, uteri obtained from Sprague–Dawley rats (100–150 g) were homogenized in Tris buffer (10 m*M*, pH 7.4) (1 uterus/2 ml), which contained EDTA (1.5 m*M*) and sodium azide (3 m*M*). The homogenate was centrifuged at 100,000*g* for 1 hr at 4°C. Uteri cytosol was then pretreated with dextran-coated charcoal as described (10). To investigate the nature of the interaction of estradiol with the estrogen receptor site, a saturation curve was obtained for [^{3}H]estradiol (10^{-5} to 10^{-10} *M*) in the presence or absence of excess estradiol (2×10^{-5} *M*). Uteri cytosol was incubated at 4°C for 16 hr with [^{3}H]estradiol (5 nmol/tube) and competitor (ranging from 2×10^{-3} to 2×10^{-8} *M*) or with estradiol (2×10^{-5} *M*) (nonspecific). The concentration of test compounds which decreased 50% (IC_{50}) of specific radioligand binding and the inhibition constant were determined (9). Protein concentrations were determined according to the method of Lowry *et al.* (12).

RESULTS AND DISCUSSION

Chemistry

N,N-Dimethyl (VII) and *N,N*-diethylfluoro (IV and VI) analogues of tamoxifen were prepared from the corresponding hydroxy analogues of tamoxifen via tosyl analogues by displacement with fluoride. The synthesis of *N,N*-diethylfluorotamoxifen and fluoromethyl-*N,N*-diethyltamoxifen has been simplified to a three-step procedure (Fig. 1). In addition, the *cis* and *trans* isomers of the desired products were separated by passing the reaction mixture through a

Table II. ^{3}H-NMR Data of Tamoxifen Derivatives

	H-1	$J_{1,2}$	$J_{1,2'}$	H-2	H-3	$J_{3,4}$	$J_{3,4'}$	H-4
II (*cis*)	2.79	6.3	6.3	3.96	2.70	7.1	7.1	3.49
II (*trans*)	2.72	6.2	6.3	3.88	2.76	7.1	7.1	3.54
III (*cis*)	≈2.48	—	6.3	3.99	≈2.64	—	7.3	1.56
III (*trans*)	≈2.45	—	6.4	3.90	2.77	6.4	7.3	1.59
VIII (*cis*)	2.91	6.3	7.1	3.94	2.84	7.1	6.3	4.07
VIII (*trans*)	≈2.80	—	—	≈3.89	≈2.76	—	—	≈3.94
IX (*cis*)	2.48	6.0	6.3	3.90	2.90	6.0	7.1	1.66

silica gel-packed column and eluting with ether/petroleum ether/triethylamine (1:1:0.1). The ^{1}H-NMR chemical shift signals for *cis* and *trans* isomers were assigned based on published information (8,11). It was ascertained that the tosyl group on *N,N*-diethyl-*O*-tosyltamoxifen could be displaced by nucleophilic fluoride substitution reaction with a milder condition (e.g., kriptofix-222 and KF). Using this procedure, the fluoro analogue of tamoxifen (IV) was prepared in 40% yield from the corresponding tosyl derivative of hydroxytamoxifen. However, elimination occurred in the presence of the stronger base (e.g., tetrabutylammoniumhydroxide). The formation of the butadiene by-product is due to an elimination reaction on the tosyl analogue. Increasing the side chain by one carbon results in the synthesis of *cis* fluoromethyl-*N,N*-diethyltamoxifen (VI), which is more stable toward tosyl elimination. The yield for compound VI was 60%.

In Vitro *Receptor Binding Assay*

For [^{3}H]estradiol binding, Scatchard analysis indicated a single class of binding sites with a mean K_d of 10 n*M* (n = 3) and a mean B_{max} of 13.6 fmol/mg protein with a Hill coefficient of 0.995. The inhibition constants (K_i) of the ligands for the estrogen receptor binding in rat uteri were then determined and their relative binding affinities (RBA) were calculated. These results are summarized in Table I. Fluorotamoxifen (VII) binds to the estrogen receptor with the same affinity as tamoxifen (IC_{50}, 500 n*M*) (Table I). The affinity of the *trans* isomer of *N,N*-diethylfluorotamoxifen for the estrogen receptor is two and a half times that of tamoxifen. In addition, the *N,N*-diethyl *trans* isomer has a higher binding affinity than the *cis* isomer. Increasing the side chain with one carbon resulted in the formation of compound VI, which showed a fivefold higher affinity for the estradiol binding site than tamoxifen. Receptor binding affinity of fluorotamoxifen with a fluorine atom placed on the phenyl ring of tamoxifen has been reported (13). However, the reaction preparation used for fluorotamoxifen can yield only low specific activity for ^{18}F-labeled tamoxifen, which is not practical for estrogen-receptor studies using PET.

In summary, this study demonstrates that fluorinated tamoxifens bind to estrogen receptors *in vitro*, thus reflecting a potential for use in imaging estrogen receptors by PET. Also, the data obtained from *In vitro* receptor assays suggested that fluoromethyl-*N,N*-diethyltamoxifen may be a potential ligand for mapping the estrogen receptor by PET.

ACKNOWLEDGMENTS

This work was supported in part by the John S. Dunn Chair in Diagnostic Radiology, the John S. Dunn Foundation, and Cancer Fighters of Houston. We thank Dianne Perez-Onuogu and LaDonna McClain for preparing the manuscript and Raquel Collins for her assistance in receptor assay studies.

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