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Loss of antagonistic activity of tamoxifen by replacement of one *N*-methyl of its side chain by fluorinated residues

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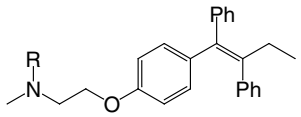
Abstract—Efforts to limit the metabolic alteration of the aminoalkyl side chain of tamoxifen by fluorination largely decrease its ER-mediated antagonistic properties in MCF-7 cells (i.e., ability to inhibit growth, to stabilize ER, and to modulate ERE and AP-1 transcriptional activity). This loss is associated with an enhancement of agonistic activity. Loss of interaction between Asp 351 and the nitrogen atom of tamoxifen provoked by the fluorination of its side chain may explain this property.

1. Introduction

Tamoxifen **1a** (Fig. 1) is now widely used for the treatment of estrogen receptor-positive (ERα form) breast cancers. Although various targets seem to be involved in the mode of action of this partial antiestrogen,[1] it is clear that it mainly acts through maintaining ER in status able to repress genes regulating tumor development.[2] Basicity of aminoalkyl side chain (i.e., $[\text{-OCH}_2\text{CH}_2\text{N(CH}_3)_2]$) of tamoxifen has clearly been shown to play a major role in this regulatory process. Chemical changes aimed to diminish the potency of the nitrogen atom of this side chain to accept hydrogen bond always lead to a decrease of antiestrogenicity.[3]



1a R = Me
1b R = H
1c R = CH_2CF_3
1d R = $CH_2CH_2CF_3$
1e R = $CH_2CH_2OCF_3$

2c R = Et
2d R = *n*Pr
2e R = *n*Bu

Figure 1. Tamoxifen derivatives used in this work.

A study devoted to limit the metabolism of tamoxifen revealed that the replacement of one *N*-methyl of its aminoalkyl side chain by a *N*-(2,2,2-trifluoroethyl) (compound **1c**) abrogates the potency of the compound to inhibit the growth of the (ER+) MCF-7 breast cancer cell line.[4] A decrease of basicity of the nitrogen atom of this side chain provoked by this chemical conversion may be taken as responsible for this property. However, this explanation is speculative since such a loss of basicity had not been reported at the time of this study. The present paper evaluates the validity of this explanation by examining a series of fluorinated derivatives of tamoxifen spanning an extended range of basicity, as well as their corresponding non-fluorinated analogues in order to detect any effect induced by the chain length of the substituent. It also provides information concerning the mechanism by which tamoxifen regulates ER-mediated growth and transcription.

2. Results

2.1. Chemical synthesis

A convergent synthesis of the target fluorinated tamoxifen derivatives was planned by N-alkylation of *N*-desmethyltamoxifen **1b**.[5] Alkylation of amine **1b** was first performed with 2,2,2-trifluoroethyl trifluoromethanesulfonate[6] (THF, NEt_3, rt, 24 h) leading to trifluoroethyl

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tamoxifen **1c** in 85% yield.[4] The preparation of the trifluoropropyl derivative **1d** proved cumbersome. Preparation, under standard conditions, of 3,3,3-trifluoropropyl trifluoromethanesulfonate resulted only in low yield (27%) of this unstable triflate.[7] Moreover upon attempted condensation with amine **1b**, no useful yield of the trifluoromethylated derivative could be isolated. However, alkylation of **1b** with 3,3,3-trifluoropropyl iodide[8] in the presence of triethylamine (THF, rt, 24 h, then reflux 24 h) was more successful leading to the desired derivative **1d** in 32% yield. The last trifluoromethoxy derivative **1e** was obtained conveniently by condensation of the corresponding triflate (CF_3OCH_2-$CH_2OSO_2CF_3$)[9] with **1b** in THF (NEt_3, rt, 48 h, 23% yield). The non-fluorinated analogues of **1c**–**1e** were obtained by conventional chemical means: compound **2c** was obtained by $LiAlH_4$ reduction of the acetyl derivative of **1b**;[10] higher homologues **2d** and **2e** were prepared via reductive alkylation of **1b** with $NaBH_3CN$ and the corresponding aldehydes.[11]

2.2. pKa measurements

The very low solubility of compounds **1c**–**1e** in pure water or in water–DMSO mixtures (up to 50% DMSO) as well as in ethanol–water precluded the determination, even by extrapolation, of pK_a by standard titrimetric methods. Measurements were thus made spectrophotometrically in pure DMSO using overlapping indicators.[12] The pK_a values for compounds **2c**–**2e** may be expected to lie very close to the value determined for **1a**.[13] The measured values are gathered in Table 1.

Although pK_a values determined in DMSO may differ from those obtained in water, this approach is nevertheless valid for amines[14] provided that a series of closely related compounds is analyzed, as is the case here. The values determined in this way reflect thus confidently the variations of pK_a in our series. The imprecise figure obtained for compound **1c** is the consequence of the lack of a suitable color indicator in this pK_a range for the method used.[15]

The influence of fluorine substitution on the basicity of neighbor amines has been sporadically examined in the literature; mainly concerning mono- or difluorinated compounds.[16] The major trend recorded is a decrease of the pK_a upon successive fluorine substitution. Ultimately a lowering of 4–5 pK_a units is to be expected for the replacement of a methyl group by a 2,2,2-trifluoroethyl group as observed for **1c**. For a trifluoropropyl substitution, a decrease in the range of 1.8 pK_a unit may be expected.[17,18] The value recorded here ($\Delta pK_a = -1.64$) for compound **1d** is in line with this observation. The behavior of the trifluoromethoxy group is less well understood, few data are available in the aromatic series but practically nothing is known for aliphatic trifluoromethylethers, which are currently rare compounds.[19] It has been advocated that the electronic properties of a trifluoromethoxy group (at least in the aromatic series) lie somewhere between those of a chlorine and a fluorine atom.[20] Based on this assumption and the value published for 2-fluoroethylamine[21] and methylamine[18] one can roughly expect a lowering of ca. 2.2 pK_a units upon γ substitution by OCF_3. The value observed here ($\Delta pK_a = -1.3$) for **1e** is lower than expected and presumably reflects the crudeness of the initial assumption. Some caution must thus be exercised when extrapolating properties observed on aromatic nucleus to aliphatic derivatives.

Table 1. pKa of the tamoxifen derivatives

Compound	pK_a (DMSO)
1a	7.70
1c	< 4
1d	6.06
1e	6.40

2.3. Biology

2.3.1. Binding affinity for ER. When tested on a pure hERα preparation, tamoxifen displayed a binding affinity for the receptor quite similar to that measured with uterine cytosols (RBA 2% of 17β estradiol (E_2)). Binding affinity of fluorinated compounds **1c**–**1e** was about one order of magnitude less than that of tamoxifen (RBA ~0.2–0.3%). This property could not be ascribed to an increase of side chain length, because non-fluorinated analogues (**2c**–**2e**) failed to show such a loss of binding affinity (they were as effective as tamoxifen in our assay). Hence, the loss of basicity of tamoxifen associated with the fluorination of its side chain would decrease the capacity of the latter to interact with amino acid residues of the ligand binding pocket that contribute to the anchorage of the compound within the receptor. Interestingly, a very high loss of basicity appeared not required to elicit such a behavior since the three investigated fluorinated compounds displayed similar RBA values.

2.3.2. Influence of fluorination of tamoxifen on its estrogenic/antiestrogenic profile. Data reported hereunder established in MCF-7 breast cancer cells clearly indicate a displacement of the antagonistic/agonistic balance of tamoxifen in favor of its agonistic activity.

2.3.2.1. Growth. Fluorinated compounds, in contrast to tamoxifen, increased the biomass of MCF-7 cells and failed to abrogate the trophic effect of estradiol (E_2 0.1 nM); tamoxifen suppressed the growth promoting effect of these compounds (Figs. 2 and 3). The relative low binding affinity for ER of these compounds was reflected upon their low trophic efficiency (stimulation at 0.1 μM). Note the absence of antagonistic activity of 1 μM tamoxifen on basal growth in these experiments. This property is due to a decrease of basal growth rate associated with the replacement of the plating medium at the time of exposure of the cells to the ligands, a condition especially appropriate for detecting weak agonistic properties of ligands. Without such a medium change, tamoxifen-induced growth inhibition was recorded, while fluorinated derivatives remained inactive (percentage of control value: **1a** = 63; **1c** = 106; **1d** = 93; **1e** = 99).

At the highest concentration tested (1 μM), **1c** appeared slightly more potent than **1d** and **1e** suggesting that the

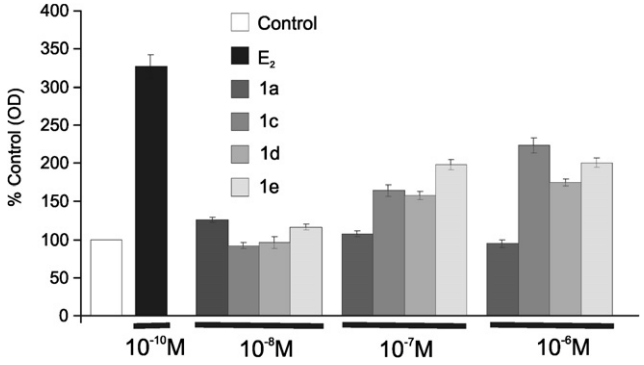

Figure 2. Growth promoting effect of fluorination of the side chain of tamoxifen. MCF-7 cells were cultured for 3 days with investigated fluorinated tamoxifen derivatives at 10^{-8} M, 10^{-7} M or 10^{-6} M. Untreated cells (control) as well as cells exposed to 10^{-10} M E_2 were cultured in parallel. Data correspond to means of 6 values ± SD (established in 2 independent experiments).

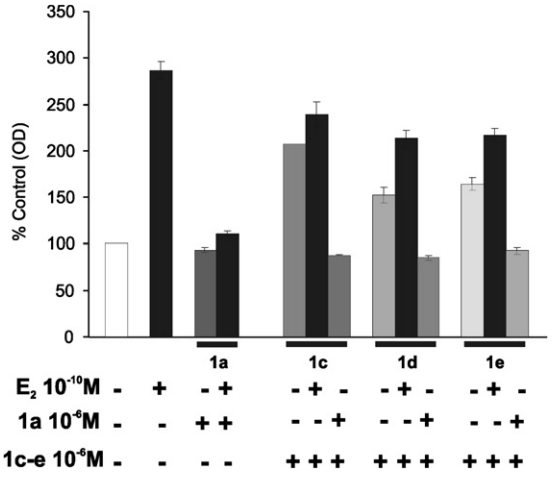

Figure 3. Effect of estradiol and tamoxifen on the growth promoting effect of fluorinated tamoxifen derivatives. MCF-7 cells were cultured for 3 days with investigated fluorinated tamoxifen derivatives at 10^{-6} M alone or in the presence of E_2 (10^{-10} M) or tamoxifen (10^{-6} M). Untreated cells were cultured in parallel as control. Data correspond to means of 6 values ± SD (established in 2 independent experiments).

loss of basicity induced by the fluorination of the side chain of tamoxifen may be directly responsible for the emergence of the present trophic activity. The assessment of non-fluorinated analogues of the three investigated compounds confirmed this statement. Thus, when tested at 1 μM in a comparative assay, these three analogues (**2c**–**e**) failed to stimulate MCF-7 cell growth, while **1c**–**e** remained active (Fig. 4). At a 10-fold higher concentration, **2c**–**e** were inhibitory and/or toxic, while **1c**–**e** were still stimulatory (percentage of control value : **1a** = 74, **2c/1c** = 65/220, **2d/1d** = 68/138, and **2c/1c** = 70/149). Hence, loss of basicity produces a growth promoting effect even if it is detrimental for ER binding.

2.3.2.2. ER level. Basicity of the side chain of tamoxifen protects ER against proteasomal degradation leading to its progressive accumulation (tamoxifen does not interfere with ER synthesis).[22] Hence, fluorination of

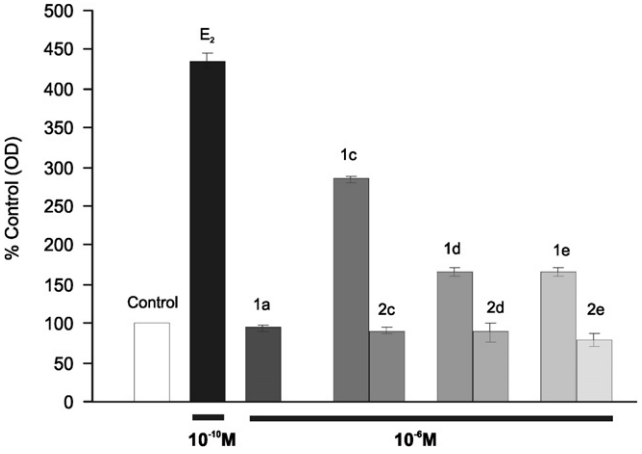

Figure 4. Implication of fluorine in the growth promoting effect of fluorinated tamoxifen derivatives. MCF-7 cells were cultured for 3 days with either fluorinated or non-fluorinated tamoxifen derivatives at 10^{-6} M. Untreated cells (control) as well as cells exposed to 10^{-10} M E_2 were cultured in parallel. Data correspond to means of 6 values ± SD (established in 2 independent experiments).

this chain would logically abrogate this protective effect. Exposure of MCF-7 cells to the three investigated fluorinated compounds (1 μM, up to 48 h of incubation) confirms this hypothesis. Thus, in contrast to tamoxifen, all derivatives failed to stabilize ER (Figs. 5 and 6) leading even to its moderate depletion after 48 hours of treatment (**1c**). This receptor loss, usually found with estrogenic ligands, was totally suppressed by tamoxifen. Although, **1c** appeared slightly more potent than **1d** and **1e** to eliminate ER after a prolonged treatment, no major influence of the degree of basicity loss was recorded.

2.3.2.3. ER-mediated transcription. Ligand-induced conformational changes of ER allow its stable association with estrogen response elements (EREs) located in the promoter regions of target genes of which it controls the expression. ER acts also as a co-regulator of other transcription factors, bound to their own response elements. Both behaviors do not necessarily give the same response. Therefore, to define the estrogenic/antiestrogenic profile of a ligand, we usually test its effect on stably transfected MCF-7 cells with a reporter gene, i.e., luciferase, respectively, under the control of ERE (MVLN cells)[23] or TRE which enhances AP-1 transcrip-

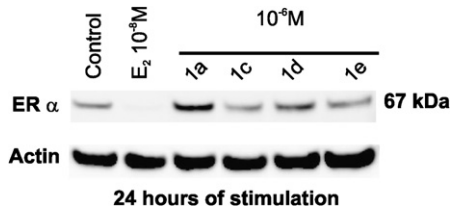

Figure 5. Influence of fluorination of tamoxifen on its ability to regulate ER level in MCF-7 cells. Cells were exposed for 24 h to either 10^{-8} M E_2 (ER down regulation), 10^{-6} M tamoxifen **1a** (ER up regulation) or the three investigated fluorinated tamoxifen derivatives **1c**–**e** at 10^{-6} M. Untreated cells (control) were maintained in culture without any ligand. Cells were then lysed and submitted to Western blotting. Data refer to one of two experiments giving identical results.

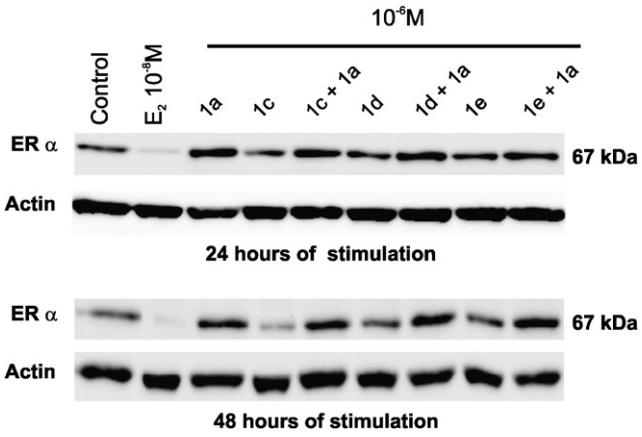

Figure 6. Ligand-induced regulation of ER level. Antagonism between tamoxifen and fluorinated tamoxifen derivatives. Cells were exposed for 24 hours or 48 hours to 10^{-8} M E_2, 10^{-6} M tamoxifen **1a** or the three investigated fluorinated tamoxifen derivatives **1c–e** at 10^{-6} M alone or in the presence of tamoxifen **1a**. Cells were then lysed and submitted to Western blotting. Data refer to one of two experiments giving identical results.

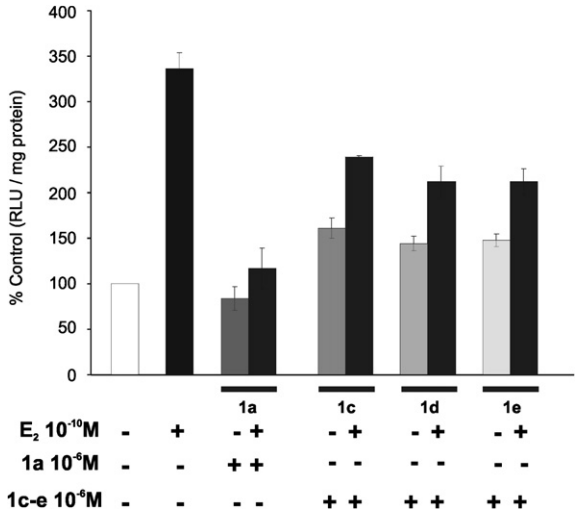

Figure 8. Influence of fluorination of tamoxifen on its ability to abrogate E_2-induced ERE-dependent transcription (MVLN cells). MVLN cells were exposed for 24 h to tamoxifen or to the investigated fluorinated tamoxifen derivatives (**1c–e**) at 10^{-6} M alone or in the presence of E_2 (10^{-10} M). Cells were then processed for luciferase measurements. Data refer to means of 4 values ± SD (established in two independent experiments).

tion under stimulation with TPA (MTLN cells).[24] With regard to the present investigation, it has been established that tamoxifen represses E_2-induced transcription in MVLN cells, while it strongly enhances the TPA-induced transcription in MTLN cells. E_2 stimulation has no effect in MTLN cells.

As shown in Figure 7, fluorination of the side chain of tamoxifen induces a slight enhancement of ERE-dependent transcription at 1 μM in MVLN cells. This displacement of the antagonistic/agonistic activity of tamoxifen concurs to a partial loss of repression of E_2-induced transcription (Fig. 8).

In MTLN cells, in contrast to tamoxifen, fluorinated tamoxifen derivatives failed to enhance TPA-induced transcription (Fig. 9). Hence, loss of basicity of the compound was reflected on both ER-mediated and -assisted transcriptions.

3. Discussion and conclusions

The present investigation clearly shows that a loss of basicity of tamoxifen provoked by the substitution of one methyl of its aminoalkyl side chain by a fluorinated residue largely decreases its ER-mediated antagonistic properties. This effect was reflected in MCF-7 cells by an enhancement of the agonistic activity on growth, ER-mediated and assisted transcriptions as well as regulation of ER level.

Crystallographic studies concerning the binding of tamoxifen to ER provide information that may explain our observations. Such studies revealed that the estrogenic (triphenylethylenic) core of the compound[25] stabilizes the hormone binding domain of the receptor in an

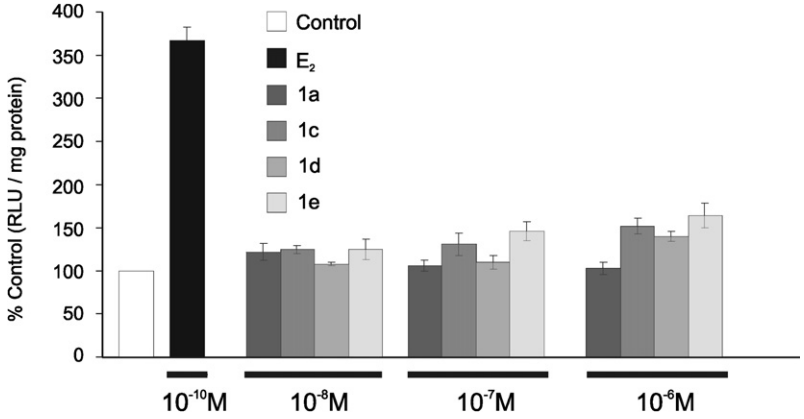

Figure 7. Influence of fluorination of tamoxifen on its ability to regulate ERE-dependent transcription (MVLN cells). MVLN cells were exposed for 24 h to tamoxifen **1a** or to the investigated fluorinated tamoxifen derivatives **1c–e** (10^{-8} M to 10^{-6} M). Untreated cells (control) as well as cells exposed to 10^{-10} M E_2 were cultured in parallel. Cells were then processed for luciferase measurement. Data refer to means of 4 values ± SD (established in two independent experiments).

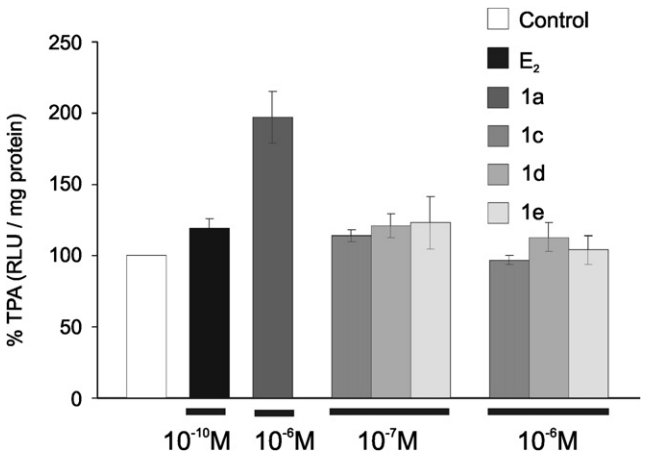

Figure 9. Influence of fluorination of tamoxifen on its ability to regulate AP-1-dependent transcription (MTLN cells). MTLN cells were exposed for 4 days to either 10^{-10} M E_2, 10^{-6} M tamoxifen **1a** or the three fluorinated tamoxifen derivatives **1c**–**e** at 10^{-7} M and 10^{-6} M. Cells were subsequently exposed for 6 h to TPA before being processed for luciferase measurement. Data refer to means of 4 values ± SD (established in two independent experiments).

'open' conformation able to recruit co-activators usually not selected by E_2, explaining thereby its weak growth promoting effect (E_2 favors a 'closed conformation' in which it is locked).[26] A key factor for this selective recruitment of co-activators is the exposure of the negative charge of an aspartate residue (Asp 351) of the hormone binding domain by triphenylethylenic estrogens. Partial neutralization of the negative charge of this amino acid by the nitrogen atom of the side chain of tamoxifen largely reduces the binding of these co-activators to the receptor leading to a decrease of gene transcription.[25,27] Hence, a loss of interaction between Asp 351 and the nitrogen atom of tamoxifen provoked by the fluorination of its side chain may explain our data. Indeed, this loss would logically increase the capacity of ER to transcribe genes, enhancing thereby cell proliferation. It may also be responsible for the weak decrease of binding affinity for ER recorded in our work.

In MCF-7 cells, E_2 enhances the proteasomal degradation of ER leading to its progressive elimination, while tamoxifen stabilizes the receptor.[22,28,29] Western blot data reported here reveal that the displacement of the antagonistic/agonistic balance of tamoxifen provoked by the fluorination of its side chain decreases the stability of the receptor indicating that estrogen-induced ER down regulation is independent of the chemical nature of the ligand. Interestingly, ER destabilization was associated with a weak increase of ERE-dependent transcription, in agreement with the concept that the proteasomal degradation of the receptor is a key step for the expression of estrogen-induced regulated genes.[28,29] Such a degradation does not seem to influence the action of transcription factors with which ER cooperates (here, AP-1 enhanced by TPA). On the contrary, our data suggest that ER stabilization (tamoxifen treatment)[22,30] enhances the potency of such transcription factors.

Agonistic activity of the three investigated fluorinated tamoxifen derivatives described here could be solely detected at high concentrations indicating a weak estrogenicity. One may anticipate that the metabolic conversion of these compounds to their 4-hydroxylated form may largely enhance their potency. The report[4] of a high binding affinity for ER of the hydroxylated form of **1c** (more than 100-fold increase in RBA value) supports this view.

Our experiments explain why efforts to limit the metabolic alteration of the aminoalkyl side chain of tamoxifen by fluorination have been unsuccessful.[4] Although the poorly known trifluoromethoxy substituent may have been a good candidate for such a purpose, the results obtained emphasize the urge for caution when extrapolating substituent properties determined on aromatic substrates to aliphatic ones. In view of the unique properties of the nitrogen atom, it seems hardly sustainable to find an atom, or a group of atoms, playing the same role. Hence, as yet, we believe that inhibition of the metabolic degradation of the side chain of tamoxifen and related SERMs remains a challenge for chemical engineering.

4. Experimental

4.1. Chemistry

NMR spectra were recorded on a Bruker AC-200 spectrometer as $CDCl_3$ solutions. Reported coupling constants and chemical shifts were based on a first order analysis. Internal reference was the residual peak of $CHCl_3$ (7.27 ppm) for ^1H (200 MHz), central peak of $CDCl_3$ (77 ppm) for ^{13}C (50 MHz) spectra, and internal $CFCl_3$ (0 ppm) for ^{19}F (188 MHz) NMR spectra. IR spectra were recorded as CCl_4 solutions on an Impact 400D Nicolet spectrophotometer. High-resolution electrospray mass spectra in the positive ion mode were obtained on a Q-TOF Ultima Global hybrid quadrupole/time-of-flight instrument (Waters-Micromass, Manchester, UK), equipped with a pneumatically assisted electrospray (Z-spray) ion source and an additional sprayer (Lock Spray) for the reference compound. Melting points were determined on a Mettler FP61 melting point apparatus. UV-Visible measurements were made at 25 °C using a HP-8353 Hewlett–Packard spectrophotometer.

4.1.1. {2-[4-(1,2-Diphenyl-but-1-enyl)-phenoxy]-ethyl}-methyl-(2,2,2-trifluoroethyl)-amine (1c). Triethylamine (70 μL, 2 equiv) and then trifluoroethyltriflate (116 mg, 2 equiv) were carefully added to a solution of *N*-desmethyl tamoxifen **1b** (80 mg, 22 mmol) in dry THF (2 mL) under argon at room temperature. The reaction was allowed to proceed for 24 h and the reaction mixture was poured into water (5 mL). The product was extracted with CH_2Cl_2 (3× 5 mL). The organic layer was washed once with a saturated $NaHCO_3$ solution (5 mL) then with water (2× 5 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was purified by chromatography (CH_2Cl_2) to

afford compound **1c** as white crystals in 85% yield. Mp: 63.7–64.1 °C; ^{1}H NMR δ 0.97 (t, J = 7.5 Hz, 3H), 2.50 (q, J = 7.5 Hz, 2H), 2.54 (s, 3H), 2.99 (t, J= 5.7 Hz, 2H), 3.17 (q, $^3J_{H-F}$ = 9.6 Hz, 2H), 3.97 (t, J = 5.7 Hz, 2H), 6.57 (d, J = 8.8 Hz, 2H), 6.82 (d, J = 8.8 Hz, 2H), 7.14–7.27 (m, 10H); ^{13}C NMR δ 13.6, 29.0, 43.2, 56.4, 58.2 (q, $^2J_{C-F}$ = 30 Hz), 65.9, 113.4, 125.7 (q, $^1J_{C-F}$ = 281 Hz), 126.0, 126.5, 127.6, 127.9, 128.1, 129.5, 129.7, 131.9, 135.8, 138.3, 141.5, 142.4, 143.8; 156.6; ^{19}F NMR δ −70.2 (t, $^3J_{H-F}$ = 10 Hz, 3F); CIMS (CH_4) m/z 440 (MH^+, 100%); Anal. Calcd for $C_{27}H_{28}NOF_3$: C, 73.78; H, 6.42; N, 3.18. Found: C, 73.75; H, 6.38; N 2.91.

4.1.2. {2-[4-(1,2-Diphenyl-but-1-enyl)-phenoxy]-ethyl}-methyl-(3,3,3-trifluoropropyl)-amine (1d). Triethylamine (116 μL, 2 equiv) and then 3,3,3-trifluoro-1-iodopropane (70 μl, 1.75 equiv) were carefully added to a solution of *N*-desmethyl tamoxifen **1b** (150 mg, 42 mmol) in dry THF (2 mL) under argon at room temperature. After 24 h stirring at rt, the reaction was refluxed for additional 24 h. The reaction mixture was poured into ice water (10 mL). The product was extracted with CH_2Cl_2 (3× 5 mL). The organic layer was washed once with a saturated $NaHCO_3$ solution (10 mL) and twice with water (2× 5 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography (CH_2Cl_2) to afford compound **1d** as white needles in 32% yield. Mp: 56.1–56.5 °C; ^{1}H NMR δ 0.95 (t, J = 7.3 Hz, 3H), 2.28 (m, 2H), 2.35 (s, 3H), 2.48 (q, J = 7.3 Hz, 2H), 2.76 (m, 4H), 3.95 (t, J = 5.7 Hz, 2H), 6.56 (d, J = 8.7 Hz, 2H), 6.80 (d, J = 8.7 Hz, 2H), 7.12–7.40 (m, 10H); ^{13}C NMR δ 13.6, 29.0, 31.3 (q, $^2J_{C-F}$ = 27 Hz), 42.5, 50.35 (q, $^3J_{C-F}$ = 3 Hz), 55.9, 65.7, 113.3, 126.0, 126.5, 127.9, 128.0, 129.3 (q, $^1J_{C-F}$ = 276 Hz), 129.4, 129.7, 131.9, 135.7, 138.2, 141.4, 142.4, 143.8, 156.6; ^{19}F NMR δ-65.7 (t, $^3J_{H-F}$ = 11 Hz, 3F); Anal. Calcd for $C_{28}H_{30}NOF_3$: C, 74.15; H, 6.67; N, 3.09. Found: C, 74.01; H, 6.74; N, 2.94.

4.1.3. {2-[4-(1,2-Diphenyl-but-1-enyl)-phenoxy]-ethyl}-methyl-(2-trifluoromethoxyethyl)-amine (1e). Triethylamine (92 μl, 2 equiv) and then trifluoromethoxyethyltriflate (90 mg, 1.1 equiv) were carefully added to a solution of *N*-desmethyl tamoxifen **1b** (120 mg, 34 mmol) in dry THF (2 mL) under argon at room temperature. After 48 h, the reaction mixture was poured into ice water (7 mL). The product was extracted with CH_2Cl_2 (3× 5 mL). The organic layer was washed once with a saturated $NaHCO_3$ solution (5 mL), and with water (2× 5 mL), dried over $MgSO_4$, and evaporated in vacuum. The crude product was purified by chromatography (CH_2Cl_2) to afford compound **1e** as crystals in 23% yield. m.p.: 51.1–51.5 °C; ^{1}H NMR δ 0.93 (t, J = 7.5 Hz, 3H), 2.38 (s, 3H), 2.43 (q, J = 7.5 Hz, 2H), 2.82 (m, 4H), 3.94 (t, J = 5.7 Hz, 2H), 4.04 (t, J = 6.0 Hz, 2H), 6.55 (d, J = 8.4 Hz, 2H), 6.78 (d, J = 8.4 Hz, 2H), 7.11–7.36 (m, 10H); ^{13}C NMR δ 13.5, 29.0, 43.0, 55.7, 56.3, 65.3 (q, $^3J_{C-F}$ = 3 Hz), 65.7, 113.3, 121.6 (q, $^1J_{C-F}$ = 254 Hz), 126.0, 126.5, 127.8, 128.0, 129.4, 129.7, 131.8, 135.7, 138.2, 141.4, 142.4, 143.8; 156.6; ^{19}F NMR δ-61.3 (s, 3F); Anal. Calcd for $C_{28}H_{30}NO_2F_3$: C, 71.62; H, 6.44; N, 2.98. Found: C, 71.66; H, 6.65; N, 2.75.

4.1.4. {2-[4-(1,2-Diphenyl-but-1-enyl)-phenoxy]-ethyl}-methyl-ethyl-amine (2c). Triethylamine (172 μL, 8 equiv) and acetic anhydride (58 μl, 4 equiv) were added to a solution of *N*-desmethyl tamoxifen **1b** (55 mg, 0.15 mmol) in dry CH_2Cl_2 (1.5 mL) under argon at room temperature. After 12 h stirring at rt, the reaction mixture was poured into ice water (3 mL). The product was extracted with CH_2Cl_2 (3× 7 mL). The organic layer was washed once with a saturated $NaHCO_3$ solution (5 mL) and twice with water (2× 5 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography (CH_2Cl_2) to afford the acetyl derivative of **1b** (51 mg) in 83% yield. ^{1}H NMR δ 0.93 (t, J = 7.34 Hz, 3H), 2.06 (s, 3H), 2.46 (q, J = 7.34 Hz, 2H), 2.96 & 3.08 (2s, NCO *cis*/*trans* ratio: 65/35, 3H), 3.66 (m, 2H), 3.99 (m, 2H), 6.52 (d, 2H), 6.78 (d, 2H), 7.12–7.36 (m, 10H); ^{13}C NMR δ 13.6, 21.4 & 21.8, 29.0, 33.7 & 38.6, 47.7 & 49.9, 64.8 & 66.4, 113.2, 126.0, 126.5, 127.8, 128.1, 129.4, 129.6, 131.9, 135.7 & 136.2, 138.0 & 138.1, 141.4 & 141.6, 142.3 & 142.4, 143.6 & 143.7, 156.0 & 156.5; MS (pos.ESI) m/z 422 (MNa^+), 400 (MH^+).

$LiAlH_4$ (9 mg, 1.85 equiv) was carefully added to a stirred solution of the previous acetate (50 mg, 0.125 mmol) in dry THF (1.25 mL). The reaction was allowed to proceed for 3 h at room temperature. Water (10 μL), a 15% NaOH aqueous solution (10 μL), and water (30 μL) were successively added under vigorous stirring. The white precipitate formed was filtered and the filtrate was concentrated under reduced pressure. Purification by silica gel chromatography of the residue (CH_2Cl_2/MeOH, 90:10) yielded amine **2c** as white solid (34 mg, 71%). Mp: 60.5–60.7 °C. ^{1}H NMR δ 0.93 (t, J = 7.34 Hz, 3H), 1.07 (t, J = 7.02 Hz, 3H), 2.30 (s, 3H), 2.49 (m, 4H), 2.73 (t, J = 6.03 Hz, 2H), 3.95 (t, J = 6.03 Hz, 2H), 6.56 (d, J = 8.56 Hz, 2H), 6.77 (d, J = 8.56 Hz, 2H), 7.12–7.40 (m, 10H); ^{13}C NMR δ 12.1, 13.6, 29.0, 42.2, 51.8, 55.7, 65.8, 113.4, 126.0, 126.5, 127.9, 128.1, 129.4, 129.7, 131.8, 135.5, 138.2, 141.3, 142.4, 143.8, 156.7; MS (pos.ESI) m/z 408 (MNa^+), 386 (MH^+); HRMS (M^+) calcd for $C_{27}H_{32}NO$: 386.2484, found: 386.2473.

4.1.5. General procedure for reductive amination of *N*-desmethyl tamoxifen 1b. The aldehyde (1.5 equiv) was added to a stirred solution of *N*-desmethyl tamoxifen **1b** (ca. 0.15 mmol) in MeOH (C = 0.1 M). After 1 h stirring at room temperature, sodium cyanoborohydride (1.7 equiv) was added and the reaction was allowed to proceed for an additional 12 h. The crude mixture was poured into water (3 mL) and CH_2Cl_2 (7 mL). The organic layer was washed with water (2 × 5 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The crude mixture was purified by silica gel chromatography (CH_2Cl_2/MeOH, 90:10) to afford the corresponding amine.

4.1.5.1. {2-[4-(1,2-Diphenyl-but-1-enyl)-phenoxy]-ethyl}-methyl-propyl-amine (2d). Yield 54%. ^{1}H NMR δ

0.93 (m, 6H), 1.64 (m, 2H), 2.45 (m, 2H), 2.56 (s, 3H), 2.72 (m, 2H), 3.06 (m, 2H), 4.07 (t, 2H), 6.54 (d, 2H), 6.79 (d, 2H), 7.12–7.35 (m, 10H); ^{13}C NMR δ 11.3, 13.5, 18.7, 29.0, 41.7, 55.2, 59.0, 63.6, 113.3, 126.0, 126.5, 127.9, 128.1, 129.4, 129.6, 131.9, 136.3, 138.0, 141.7, 142.2, 143.6, 155.7; MS (pos.ESI) *m/z* 422 (MNa^+), 400 (MH^+); HRMS (M^+) calcd for $C_{28}H_{34}NO$: 400.2640, found: 400.2646.

4.1.5.2. {2-[4-(1,2-Diphenyl-but-1-enyl)-phenoxy]-ethyl}-methyl-butyl-amine (2e). Yield 61%. ^{1}H NMR δ 0.91 (q, J = 7.50 Hz, 6H), 1.29 (m, 2H), 1.45 (m, 2H), 2.29 (s, 3H), 2.41 (m, 4H), 2.72 (t, J = 6.03 Hz, 2H), 3.94 (t, J = 6.03 Hz, 2H), 6.54 (d, J = 8.64 Hz, 2H), 6.77 (d, J = 8.64 Hz, 2H), 7.12–7.38 (m, 10H); ^{13}C NMR δ 13.6, 14.0, 20.6, 29.0, 29.3, 42.9, 56.1, 58.0, 65.8, 113.3, 126.0, 126.5, 127.9, 128.1, 129.4, 129.7, 131.9, 135.5, 138.2, 141.3, 142.4, 143.8, 156.7; MS (pos.ESI) *m/z* 436 (MNa^+), 414 (MH^+); HRMS (M^+) calcd for $C_{29}H_{36}NO$: 414.2797, found: 414.2806.

4.1.6. pKa measurements. We used a spectrophotometric method,[10] taking as indicators 2,4,6-trinitrophenylaniline for the pKa determination of tamoxifen **1a**, 2,4-dinitrophenol for compound **1c**, and 2,2′,4,6-tetranitrodiphenylaniline for both **1d** and **1e**. In a typical experiment spectra were recorded with a constant concentration (5×10^{-5} mol dm^{-3}) of indicator in buffered solutions with the amine and its amine salt (ca. 10^{-3} M). DMSO used was of the purest commercially available quality and distilled under vacuum over calcium hydride prior to use.

4.2. Biology

Stock solutions of all investigated compounds (solvent ethanol) were diluted in buffer (RBA measurement) or medium (cell culture) to maintain solvent at a minimal concentration below 0.1%.

4.2.1. Binding affinity for ER. Highly purified recombinant hERα (Calbiochem, Euro-Biochem, Bierges, Belgium) diluted in a bovine serum albumin solution (1 mg/ml) was adsorbed onto hydroxylapatite (HAP). After removal of unbound material by centrifugation, HAP was incubated overnight at 0–4 °C with 1 nM [^{3}H]E_2 (Amersham Biosciences, Roosendaal, NL) in the presence or absence of increasing amounts of either E_2 (Sigma, Saint-Louis, MO) or tamoxifen used as references, either of the investigated fluorinated tamoxifen derivatives. Radioactivity adsorbed onto HAP was then extracted with ethanol and measured by liquid scintillation counting. Relative concentrations of E_2 and investigated compound required to reduce the binding of [^{3}H]E_2 by 50% gave the relative binding affinity (RBA); RBA = ([I_{50}] compound/[I_{50}]E_2) × 100.

4.2.2. Proliferation assay.[31] MCF-7 cells were seeded in 96-wells microtitration plates (plating density 2000 cells/well) in 10% fetal calf serum depleted of endogenous steroid by dextran-coated charcoal (DCC) treatment. Twenty-four hours later, medium was removed and replaced by a fresh medium containing either tamoxifen or the investigated fluorinated tamoxifen derivatives for three additional days; control cells were maintained in culture without any compound or exposed to 0.1 nM E_2. At the end of incubation, cells were gently washed once with PBS, fixed with 1% glutaraldehyde/PBS (15 min, 20 °C), and stained with 0.1% crystal violet/H_2O (30 min, 20 °C). Excess of crystal violet dye was then removed by three washes of running tap water (15 min, 20 °C) and cells were lysed with 1% Triton X-100/H_2O (90 min, 20 °C, under agitation). Absorbance was measured at 550 nm using Microplate Autoreader EL309.

4.2.3. Regulation of ERα level. MCF-7 cells were plated in 100 mm ∅ Pétri–dishes (500,000 cells per dish) in 10% DCC treated fetal calf serum. After 3 days of culture, medium was removed and the cells were exposed to the investigated fluorinated tamoxifen derivatives with or without tamoxifen for 24 or 48 h in a fresh medium (all compounds at 1 μM); control cells were maintained in culture without any compound (Control) or exposed to 10 nM E_2. Cell cultures were then washed with TBS (50 mM Tris, pH 7.5, 150 mM NaCl) and lysed for 30 min at 4 °C in a lysis buffer (TBS with 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM NaF, 0.1mM orthovanadate, 0.6 mM PMSF, and 0.3 mM TPCK). Lysates were clarified by fine needle aspiration and sonication (5 min at 4 °C) followed by a centrifugation (13,000*g*, 20 min, 4 °C). The protein concentration of each sample was determined using a BCA protein assay kit (Pierce, Rockford, I ll.) After addition of loading buffer (LDS Sample 4 × buffer from In Vitrogen, Carlsbad, Ca.), proteins were boiled for 5 min. Each sample (15 μg) was then loaded onto 4–12% SDS polyacrylamide gel and subsequently, electrotransferred onto a nitrocellulose membrane (Amersham Biosciences, Roosendaal, NL). Non-specific sites were blocked with 5% non-fat dry milk in TBS–0.05% Tween 20 (3 h, room temperature). ER detection was performed with a mouse primary antibody (D-12, 1:750 dilution, overnight, 4 °C (Santa Cruz Biotechnology, Santa Cruz, Ca)) and a control anti-actin antibody (1:5000 dilution, overnight, 4 °C (Chemicon, Temecula, Ca.)). ER and actin bands were visualized with a peroxidase-labeled goat anti-mouse secondary antibody (1:1000 dilution, 2 h, room temperature) and a SuperSignal West Pico Chemiluminescent Substrate from Pierce (Rockford, I ll.).

4.2.4. Regulation of ERα transcriptional activity. MVLN cells (stably transfected MCF-7 cells with a pVit-tk-Luc reporter plasmid)[23] were cultured for 3 days in 6-well plates (plating density 100,000 cells/dish) in 10% DCC treated fetal calf serum. Medium was then removed and replaced by a fresh medium containing tamoxifen or the investigated fluorinated tamoxifen derivatives; control cells were maintained in the absence of any compound or exposed to 0.1 nM E_2. After 24 h of incubation, cells were processed for luciferase measurement. For that purpose, medium was removed and the cell monolayer washed twice with PBS. A 250 μl of a 5-fold diluted lysis solution (Promega E153A) was then added to the wells and maintained under mild agitation for 20 min to extract luciferase. Lysed cells were subse-

quently detached with a scraper and centrifuged for 5 s at 13,000*g* to clarify the extracts. Finally, 20 μl of extracts was mixed at room temperature with 100 μl of a luciferase assay reagent (Promega E151A/E152A) prepared according to the manufacturer's protocol. Induced-light was measured with a Berthold luminometer (Lumat LB 9507) and expressed in relative light units (RLU). Protein content of each extract was measured by the BCA assay (Pierce, Rockford, I ll.) and the data were expressed in mg protein.

MTLN cells (stably transfected MCF-7 cells with a $(TRE)_3$-tk-Luc reporter gene)[24] were cultured for 4 days in 3% fetal calf serum, either in the absence (control) or presence of ligands. After removal of the medium, cells were exposed to 30 nM TPA for 6 h before being processed for luciferase measurement.

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