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Genomic action of permanently charged tamoxifen derivatives via estrogen receptor-α

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ABSTRACT

Tamoxifen is a selective estrogen receptor modulator widely used in oncology and reproductive endocrinology. In order to decrease its non-desirable effects and elucidate mechanisms of action, permanently charged tamoxifen derivatives (PCTDs) have been reported. Whether PCTDs have genomic effects remains controversial. Since the clinical relevance of tamoxifen, the necessity to have new anticancer drugs, and in order to gain insights into the mechanisms of action of PCTDs, we obtained six quaternary ammonium salts derived from tamoxifen including three new compounds. We characterized them by nuclear magnetic resonance, X-ray diffraction, electron microscopy, and/or high performance liquid chromatography, and detected them in cell lysates by liquid chromatography coupled to mass spectrometry. We evaluated their binding to estrogen receptor-α (ERα, their effect on the transcriptional activity mediated by ERα (gene reporter assays), and the proliferation of cancer cells (MCF-7 and cells from a cervical cancer primary culture). Structural studies demonstrated the expected identity of the molecules. All PCTDs did bind to ERα, one of them induced ERα-mediated transcription while two others inhibited such genomic action. Accordingly, PCTDs were detected in cell lysates. PCTDs inhibited cell proliferation, noteworthy, two of them displayed higher inhibition than tamoxifen. Structure–activity analysis suggests that PCTDs permanent positive charge and the length of the aliphatic chain might be associated to the biological responses studied. We suggest genomic effects as a mechanism of action of PCTDs. The experimental approaches here used could lead to a better design of new therapeutic molecules and help to elucidate molecular mechanisms of new anticancer drugs.

1. Introduction

Tamoxifen (IUPAC name 2-[4-[(*Z*)-1,2-diphenylbut-1-enyl]-phenoxy]-*N*,*N*-dimethylethanamine), is a triphenylbutylene derivative, widely used for the treatment and prevention of estrogen-dependent breast cancer.[1] It is a selective estrogen receptor modulator (SERM), displaying estrogen receptor (ER) agonist and antagonist activities.[2] Since tamoxifen has also effects in patients with negative ER breast cancer,[3] other mechanisms of action and targets not related to ER have been suggested. Some examples of such different targets/mechanisms include inhibition of natural killer cells activity,[4] inhibition of protein kinase C,[5] changes in insulin-like growth factor levels,[6] anti-angiogenic activity,[7] as well as several effects on different ion channels.[8–18]

Several groups have made chemical modifications to the molecule, obtaining permanently charged tamoxifen derivatives (PCTDs, including tamoxifen ethyl bromide, tamoxifen methyl iodide, and tamoxifen butyl bromide), expected to not to cross the cell membrane.[8,13,14,19–21] It was proposed that the permanent charge would eliminate the intracellular effects of tamoxifen, remaining only the extracellular actions of PCTDs. Nevertheless, other studies have suggested that PCTDs are able to enter the cells without crossing the blood–brain barrier, in such a manner that these compounds might retain their action in peripheral tissues and organs, but reducing the side-effects in the central nervous system.[22–26]

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Thus, since the relevance of tamoxifen in oncology, the necessity to have new anticancer drugs, and in order to gain insights into the mechanisms of action of PCTDs, we obtained an analogue series of six quaternary ammonium salts derived from tamoxifen including three not previously reported, characterized their molecular structure, detected them in cell lysates and studied their effect on cell proliferation and transcriptional activity mediated by ERα.

2. Materials and methods

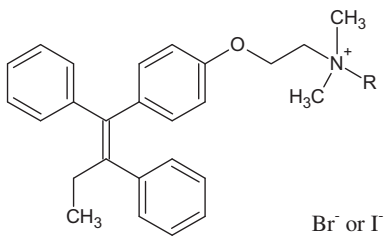
2.1. Chemical modifications

Tamoxifen free base, alkyl halides and inorganic salts, were purchased from Sigma–Aldrich Chemical Company, (San Louis, MO, USA); methanol and acetonitrile HPLC grade, used for LC/MS system, were obtained from J. T. Baker (NJ, USA). The analogue series of permanently charged tamoxifen derivatives (PCTDs, Table 1) was obtained as previously reported[26] as follows: (A) *Tamoxifen methyl iodide*, 0.5 g of tamoxifen were added to 15 ml of methyl iodide and stirred at 0 °C for 10 min, the white precipitate was filtered and recrystallized with methanol; (B) *Tamoxifen methyl bromide*, an anion interchange reaction (bromide instead of iodide) was performed, and we obtained tamoxifen methyl bromide derivative. The presence of the bromide anion was corroborated by electron microscopy (with a JEOL JSM-6300 microscope, USA) and the energy dispersion spectrometry X-ray technique (X-ray detector for energy dispersion Noral Voyager II, software 1100/1110). (C–E) *Tamoxifen ethyl bromide, tamoxifen propyl bromide,* and *tamoxifen butyl bromide*: 1 g of tamoxifen was added to 20 or 25 ml of the alkyl halide (ethyl bromide, propyl bromide and butyl bromide, respectively) and stirred at room temperature for 5 h. The white precipitate was filtered and recrystallized with methanol. (F) *Tamoxifen Isopropyl bromide* was obtained by refluxing 1 g of tamoxifen in 20 ml of isopropyl bromide for 72 h at 55 °C. The white precipitate was recrystallized with a mixture of methanol–ethyl acetate. To our knowledge tamoxifen isopropyl bromide, tamoxifen methyl bromide and tamoxifen propyl bromide are new compounds not previously reported.

2.2. Structural studies

The identity of the compounds was determined by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) on Varian Mercury-300 (USA) spectrometers at 300 and 75.4 MHz, respectively, chemical displacements were measured in ppm (δ) relative to internal tetramethylsilane.

Table 1
Aliphatic chains substituted, melting points and yield of the permanently charged tamoxifen derivatives



Compound	–R	Melting point (°C)	Yield (%)
T. methyl iodide	$-CH_3$	248–250	94.9
T. methyl bromide	$-CH_3$	168–170	80.0
T. ethyl bromide	$-CH_2-CH_3$	158–160	99.1
T. propyl bromide	$-CH_2-CH_2-CH_3$	196–198	90.1
T. isopropyl bromide	$-CH-(CH_3)_2$	198–200	37.2
T. butyl bromide	$-CH_2-CH_2-CH_2-CH_3$	176–178	94.8

T. = Tamoxifen.

2.2.1. Structural data from NMR

Tamoxifen propyl bromide: ^{1}H NMR (DMSO): δ 0.82 (t, J = 7.33 Hz, 3H, CH_3CH_2C), 0.84 (t, J = 7.17 Hz, 3H, CH_3CH_2 CH_2N), 2.39 (q, J = 7.47 Hz, 2H, CH_3CH_2), 3.05 [s, 6H, $N(CH_3)_2$], 3.25 (q, J = 7.32, 2H, $NCH_2CH_2CH_3$), 3.66 (t, J = 4.84 Hz, 2H, CH_2CH_2N), 4.3 (at, J = 4.1 Hz, 2H, OCH_2CH_2), 6.66 (d, J = 8.9 Hz, 2H, $C_6H_4OCH_2$, *meta* H), 6.73 (d, J = 8.74 Hz, 2H, $C_6H_4OCH_2$, *ortho* H), 7.1–7.4 (m, 10H, 2Ph).

Tamoxifen isopropyl bromide: ^{1}H NMR (DMSO): δ 0.83 (t, J = 7.4 Hz, 3H, CH_3CH_2C), 1.28 [d, J = 6.5 Hz, 6H, $C(CH_3)_2$], 2.37 (q, J = 7.4 Hz, 2H, CH_3CH_2), 3.0 [s, 6H, $N(CH_3)_2$], 3.69 (t, J = 4.89 Hz, 2H, CH_2CH_2N), 3.77 (m, J = 6.5 Hz, 1H, $NCHCH_3$), 4.29 (at, J = 4.73 Hz, 2H, OCH_2CH_2), 6.67 (d, J = 8.8 Hz, 2H, $C_6H_4OCH_2$, *meta* H), 6.77 (d, J = 9 Hz, 2H, $C_6H_4OCH_2$, *ortho* H), 7.1–7.4 (m, 10H, 2Ph). The rest of the compounds have been already reported.

Melting points (mp) were determined using an electro thermal J.T.R.-TEMSA melting point apparatus, values were not corrected (Table 1). Chemical purity was assessed using high performance liquid chromatography tandem to mass spectrometry (HPLC–MS).

X-ray diffraction from electron microscopy in a Bruker-Nonius CAD4 diffractometer (USA) with Cu-K_α radiation was used in order to determine the crystal structure of tamoxifen methyl bromide. SHELXS96 and SHELX97 software (University of Göttingen, Germany) were used in order to solve and refine these structures.

2.3. Binding and transcriptional studies

2.3.1. Estrogen receptor binding experiments

[2,4,6,7-^{3}H] estradiol ([^{3}H]-E_2), sp. act. 72 Ci/mmol was purchased from NEN Research Products (Boston, MA, USA), non-radioactive estradiol was supplied by Sigma–Aldrich, Inc. (St. Louis, MO, USA), and the anti-estrogen ICI 182, 780 from Zeneca Pharmaceuticals (Wilmington, DE). Cell culture media and reagents were purchased from Invitrogen life technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) was supplied by Hyclone Laboratories, Inc. (Logan, UT, USA). All reagents and solvents used were of analytical grade. We used an expression vector for human ERα (pCMV5-hERα) containing the coding sequence of the ERα and the estrogen responsive reporter plasmid (ERE-E1b-Luc). The relative receptor binding affinities for ERα were determined as previously described.[27,28] Briefly, cervical cancer HeLa cells (obtained from American Type Culture Collection, Manassas, VA) were transfected with 5 μg of ERα expression vector (pCMV5-hERα) using PolyFect (QIAGEN Inc. Valencia, CA, USA) according to the manufacturer's protocol. Forty eight hours later, cells were harvested and the cytosolic fraction was obtained in TEDLM buffer (20 mM Tris–HCl, pH 7.4, 1.5 mM EDTA, 0.25 mM dithiothreitol, 10 mg/ml leupeptine, and 10 mM sodium molibdate). The cytosolic fractions (0.2 mg protein/ml) were incubated with 1 nM [^{3}H]-E_2 and increasing concentrations (0.005–5000 nM) of radioinert estradiol, tamoxifen, or the newly synthetized PCTDs for 18 h at 4 °C. Free steroid was separated from receptor-bound steroid by addition of dextran-coated charcoal suspension (250 mg Norit-A and 25 mg Dextran T-70) in TEDLM buffer and incubated for 10 min at 4 °C. Following centrifugation (800 g at 4 °C for 15 min), bound [^{3}H]-E_2 was quantified by liquid scintillation counting (Betamax (INC, Biomedicals Inc, CA, USA). The results are expressed as the relative binding affinities (RBA) and the inhibition constants (K_i) of steroid competitors, as previously described.[27,28]

2.3.2. Transcriptional activity (luciferase assays)

HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM-HG) without phenol red, supplemented with 5%

stripped fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin; and incubated in 5% CO2 at 37 °C. The next day cells were co-transfected with 25 ng pCMV5-hERα, 20 ng TK-Renilla Promega Corp (Madison, WI, USA) and 1 μg of a luciferase reporter vector (ERE-E1b-Luc) The transfections were performed in triplicate using PolyFect (Qiagen Inc. Valencia, CA, USA), according to the manufacturer's protocol. The plates were incubated for 48 h at 37 °C in 5% CO_2. After incubation, media were replaced with DMEM-HG containing the compounds of interest and cells were incubated for 48 h. Ethanol alone was used as vehicle. Luciferase activity was then measured with the Dual Luciferase Kit Promega Corp (Madison,WI, USA) according to the manufacturer's recommendations. Firefly luciferase activity was normalized to the internal transfection control provided by the Renilla luciferase activity.

2.4. Detection of PCTDs in cell lysates

2.4.1. Preparation of lysates

HeLa cells were cultured in DMEM without phenol red, and supplemented with 10% fetal calf serum and penicillin/streptomycin (1%). Next day, cells were incubated with the corresponding compound for 48 h. After treatment, the cells were washed 10 times with PBS, trypsinized—and centrifuged 15 min at 3000 rpm. Then, cells were resuspended with PBS, centrifuged (three times), and sonicated in distilled water. Finally, a mixture of ethanol–acetonitrile was added, and the sample was centrifuged 20 min at 15,000 rpm.

2.4.2. LC/MS system

Cell lysates were analyzed with a 1100 Rapid Resolution system (Agilent Technologies USA) containing a binary pump and degasser, a well plate auto sampler with thermostat, and a thermostatted column compartment, coupled to an Agilent 6300 ion trap quadrupole mass spectrometer system equipped with an electro spray ionization (ESI) source. The separation was carried out with a Waters C18 reverse-phase column, 5 μm, and 4.6 mm × 150 mm analytical column with a mobile phase 85% (v/v) methanol in water (HPLC purity) at a flow rate of 1 ml/min, and injection volume of 25 μl at 30 °C of column temperature. All spectra were acquired in the positive ion mode, over a mass range of m/z 300–550 at rate of one scan every 2 s. The nebulizer was set to 10–15 psi and the nitrogen drying gas was set to a flow rate of 4 l/min; drying gas temperature was maintained at 325 °C.

2.5. Proliferation studies

2.5.1. [^{3}H]-thymidine incorporation

Cell proliferation assay was performed with human cervical cancer cells obtained from previously established primary cultures,[29] which were grown in plates with DMEM with 10% FCS penicillin/streptomycin (200 μg/ml). Cells were seeded in 24-well plates, until 60% confluence of more was reached. Cells were washed and incubated during 24 or 48 h with PCTDs (5 nM or 2 μM) which were dissolved in methanol as vehicle (final concentration 0.1% v/v). Four hours before completing the treatment, ^{3}H thymidine (0.5 μCi/well) was added. ^{3}H thymidine was eliminated of culture medium and radioactivity was measured as disintegrations per minute (dpm) with a Beckman Coulter LS 6500 multi purpose scintillation counter (USA).

2.5.2. MTT assay

The human breast adenocarcinoma cell line MCF-7 was obtained from American Type Culture Collection (Manassas, VA) and cultured according to manufacturer's instructions. Cells were grown in RPMI-1640 culture medium with fetal calf serum (10%), penicillin/streptomycin (1%) and non essential amino acids (1%). Cells were seeded in 96 well plates at 37 °C in a humidified atmosphere of 5% CO_2 and incubated until 60% confluence of more was reached. Cells were incubated during 96 h with PCTDs (6 μM) which were dissolved in methanol as vehicle (final concentration, 0.1 v/v%). Cell proliferation was assayed by the colorimetric method based on the conversion of the tetrazolium salts to formazan crystals by dehydrogenase activity in active mitochondria (MTT Cell proliferation Kit I, Boehringer Mannheim GmbH, Germany). MTT (0.5 mg/ml) was added to the cell culture 4 h before completing the PCTDs incubation time. Optical density data were obtained from the resulting colored solution with a microplate photometer (Sunrise Touchscreen, Tecan, USA).

2.6. Statistical analysis

Experiments were repeated at least three times. Comparisons were carried out by one-way analysis of variance (ANOVA) followed by Dunnett's test, or a two tailed t-test, differences were considered statistically significant when P <0.001 or 0.005, respectively. Analysis was made with the software Sigma Stat version 3.5 (U.S.A. or Germany).

2.7. Structure–activity relationship

The association between different parameters was analyzed using *Origin Pro v. 7.0383* 2002 (Sony Electronics, Inc.: Northampton, USA). Log P values were calculated with ALOGPS 2.1.[30]

3. Results

3.1. Chemical modifications and identity

Six PCTDs were obtained as described in Section 2. Table 1 shows the aliphatic chain substituted, yield and the resulting melting point of each compound. We obtained the expected molecular identity of each PCTD. For illustration purposes, structural analysis of only the three new, not previously reported compounds is shown. Figure 1A shows the X-ray elucidation of tamoxifen isopropyl bromide displaying the bromide atom. X-ray energy dispersion spectrometry shows the expected identity of tamoxifen methyl bromide (Fig. 1B), and ^{1}H nuclear magnetic resonance (NMR) analysis shows the identity of tamoxifen propyl bromide (Fig. 1C), using DMSO as solvent.

3.2. Presence of PCTDs in lysates from HeLa cells incubated with the compounds

In order to reveal potential intracellular effects of PCTDs, a very important issue to solve was to know if the different PCTDs could be detected in lysates from cells incubated with the compounds. High performance liquid chromatography coupled to mass spectrometry was used for detection. In order to have reliable evidences about the presence and molecular identity of PCTDs, several parameters were considered, namely, retention time value, mass–charge ratio, and fragmentation pattern (this pattern must be the same in a specific molecule, when fixing relative collision energy is applied, 2.0 V in this case).

Figure 2A shows a cell lysate chromatogram from untreated HeLa cells. No endogenous peaks that could interfere with the detection of tamoxifen derivatives were observed. Next, we determined the different parameters of a tamoxifen butyl bromide standard solution. Figure 2B shows the retention time value, m/z ratio and fragmentation pattern of tamoxifen butyl bromide standard solution. Once we had these controls, HeLa cells were incubated with the compound during 48 h and the same parameters were

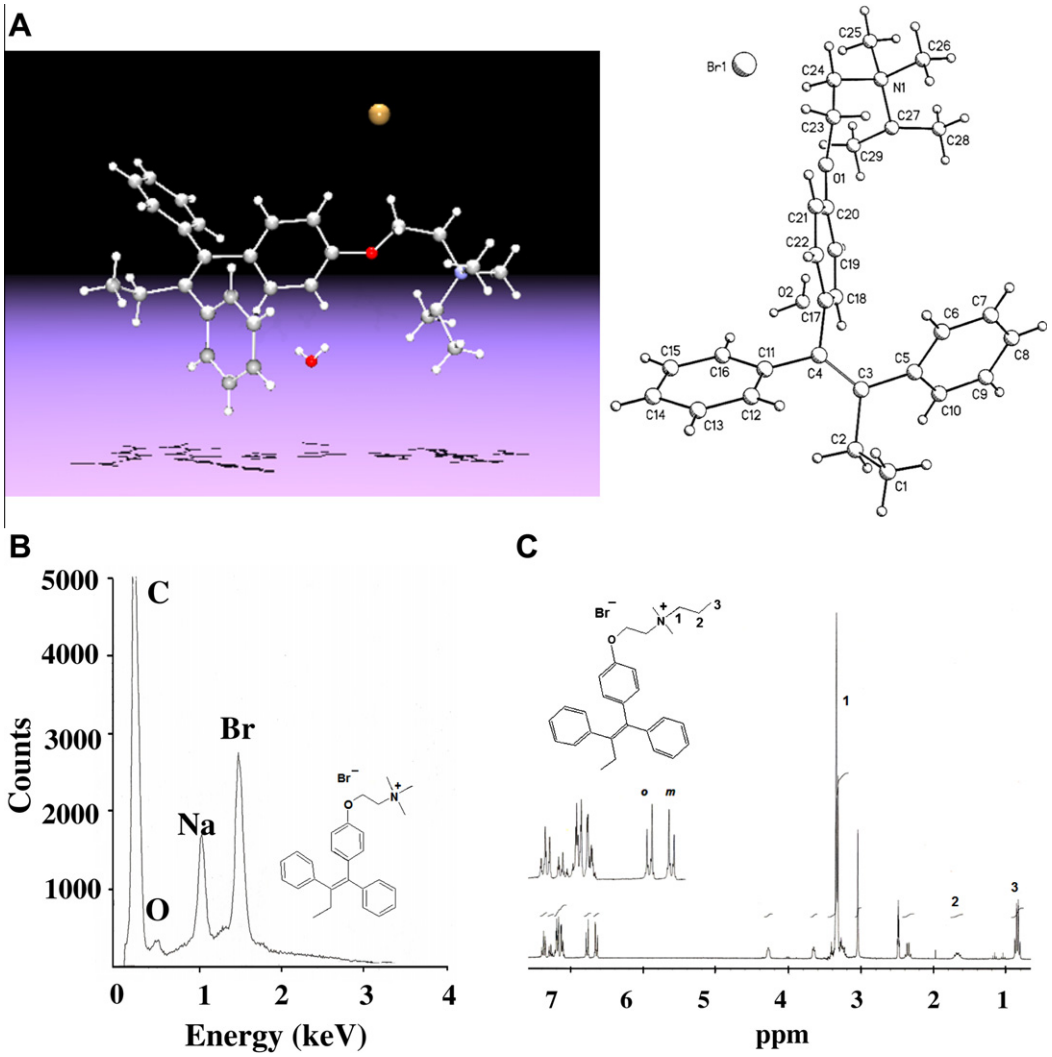


Figure 1. Structural elucidation. (A) Crystal structure analysis by X-ray diffraction of tamoxifen isopropyl bromide (left panel). C, O, N, and Br atoms are indicated in the right panel. (B) X-ray energy dispersion spectrometry of tamoxifen methyl bromide, showing the presence of the bromide atom, replacing the iodide atom. (C) Tamoxifen propyl bromide structure elucidation by ^{1}H nuclear magnetic resonance analysis showing the identity of the propyl group.

studied in the cell lysates to know whether the compound had entered the cells. Retention time value, *m*/*z* ratio and fragmentation pattern found in this cell lysate (Fig. 2C) strongly suggest the presence of tamoxifen butyl bromide inside the cells.

A summary of retention time and *m*/*z* values from the standard solutions of all PCTDs as well as PCTDs detected in the cell lysates are listed in Table 2. The values obtained for the standard solutions using the compounds in the micromolar range, show high purity of PCTDs and the absence of contamination with possible unmodified tamoxifen. Besides, all of the parameters indicate that all the PCTDs obtained enter the cells emphasizing that the compounds might have relevant intracellular effects. HPLC–MS analysis showed the expected *m*/*z* values and the absence of a tamoxifen signal in the PCTDs (Fig. 3), indicating high purity of the compounds, which is a very important issue in the study and interpretation of the biological assays.

3.3. PCTDs bind to ERα and inhibit its transcriptional activity

Since tamoxifen exerts major antiproliferative effects through its binding to ERα, we wondered whether PCTDs maintained the ability to bind to this receptor. Both tamoxifen methyl iodide and tamoxifen ethyl bromide have been already reported to bind to estrogen receptor.[26] We selected only some PCTDs for these studies because PCTDs with substituted methyl differ only in the anion, having the same cationic molecule. On the other hand, the assessment of isopropyl bromide was aimed to observe the effect of the branched chain on the affinity, instead of the effect of the linear chain. All of the PCTDs studied did bind to ERα. Figure 4A shows that increasing concentrations of PCTDs decrease the binding of labeled estradiol to ERα. Inhibition constant (K_i) and Relative Binding Affinity (RBA) values for each compound are given in Table 3. Detection of PCTDs in cell lysates from treated cells and binding to ERα strongly suggested that these compounds might have a relevant intracellular activity. Thus we performed gene reporter assays in HeLa cells transfected with human ERα. If the compounds had an intracellular activity at the transcriptional level, then the activity of the reporter gene should be affected. Activity induced by estradiol (10 nM) alone was normalized to 100% (Fig. 4B). Tamoxifen and most of the PCTDs alone (100 nM) induced only a modest activity very similar to that induced by either the vehicle or progesterone (used as a negative control). However, tamoxifen isopropyl bromide induced up to 50% of the activity (Fig. 4B), suggesting its role as a new potential ERα-agonist. As expected, transcriptional activity induced by estradiol was decreased in the presence of either tamoxifen or the antiestrogenic ICI 182, 780

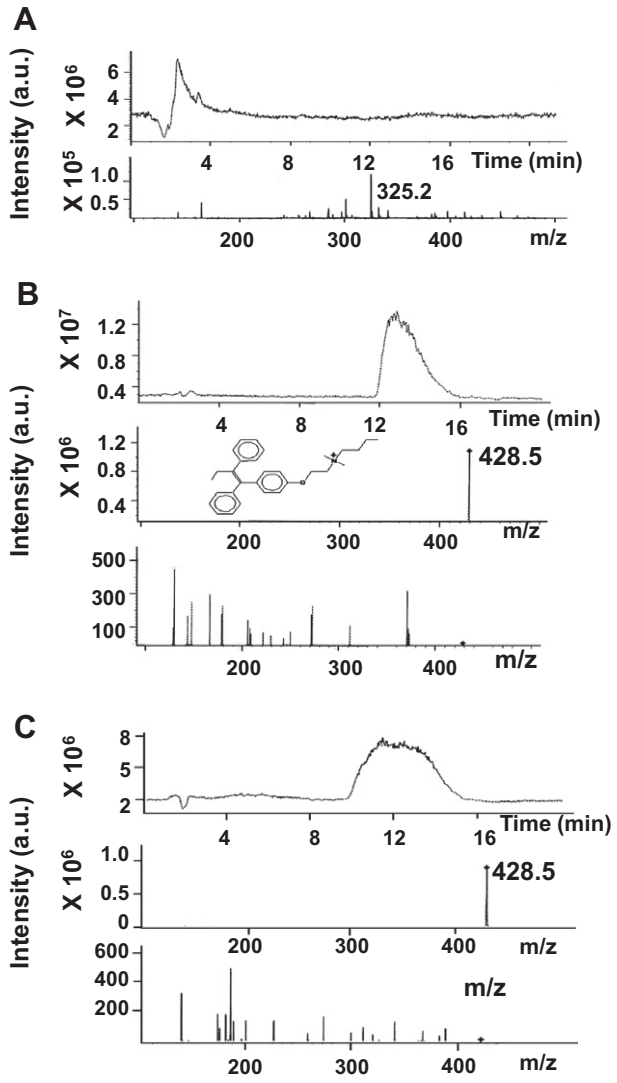


Figure 2. HPLC–ESI MS analysis of tamoxifen butyl bromide in cell lysates. (A) Cell lysates from untreated cells shows no endogenous signal potentially interfering with the detection of PCTDs. (B) Retention time, total ion chromatography (TIC), and pattern of fragmentation of tamoxifen butyl bromide standard solution (200 nM, compound diluted in methanol). (C) Same parameters as in (B) but from HeLa cell lysates incubated with tamoxifen butyl bromide (48 h, 2 μM).

Table 2
PCTDs retention times and *m*/*z* values from either standard solutions or treated HeLa cell lysates (determined by HPLC tandem mass spectrometry analysis)

Compound	Standard solutions	HeLa Cell lysates		
	Retention time (min)	$[M+H]^+$ *m*/*z*	Retention time (min)	$[M+H]^+$ *m*/*z*
Tamoxifen	8–10	372.3	9–12	372.3
Tamoxifen methyl bromide	5–8	386.3	5–10	386.3
Tamoxifen ethyl bromide	8–10	400.5	8–12	400.5
Tamoxifen propyl bromide	8–12	414.5	9–13	414.5
Tamoxifen isopropyl bromide	11–14	414.5	10–13	414.5
Tamoxifen butyl bromide	12–16	428.5	10–15	428.5

(Fig. 4C). Then we tested the effects of the two compounds with lower theoretical lipophilicity than tamoxifen, namely, tamoxifen methyl bromide and tamoxifen methyl iodide (Log *P* calculated with the software ACD/Labs 11.0, Canada). Interestingly, both compounds (1 μM) also reduced significantly the transcriptional activity induced by estradiol (Fig. 4C). All of these results propose that PCTDs might influence cell physiology by binding to ERα and affecting transcriptional activity.

3.4. PCTDs decrease proliferation of cancer cells

Finally, we wondered whether proliferation of cancer cells known to express ERα, might be affected by PCTDs. A breast cancer cell line (MCF-7) and cells from a previously established cervical cancer primary culture were used. The effect of PCTDs on cell proliferation at different concentrations (5 nM, 2 μM, and/or 6 μM) as well as different incubation times (24, 48 and 96 h) was tested. These incubation conditions (concentration and treatment duration) were selected after some experiments with 1 nM or 1 μM concentrations, in which no effect was observed. As shown in Figure 5A, all of the compounds decreased breast cancer cell proliferation (assessed by a metabolic activity assay) in comparison to untreated cells (Vehicle). Interestingly, tamoxifen isopropyl bromide and tamoxifen butyl bromide decreased cell proliferation more than tamoxifen. Cervical cancer cells from a primary culture previously characterized to express human papilloma virus oncogenes, cytokeratins, and ERα,[29,31] were also studied. Both tamoxifen and PCTDs decreased thymidine incorporation only at the lowest concentration (5 nM, Fig. 5B). The potential mechanisms explaining the effect of PCTDs on cancer cell proliferation might be related to the inhibition of the transcriptional activity mediated by ERα.

3.5. Structure–activity relationship

In order to gain some insight into structure–activity relationship, we analyzed the charge and length of the aliphatic chain of PCTDs and their effect on cell proliferation and binding to ERα (K_i and RBA), finding interesting trends. Regarding metabolic activity (Fig. 6A), the values are grouped into two clusters when plotted against calculated Log *P* (from 4-OH tamoxifen, data not shown). The difference between these two groups is the positive charge of the nitrogen of PCTDs but absent in tamoxifen. This charged nitrogen, plus the length increase on the hydrocarbonated chain, seems to enhance the activity on MCF-7 cells. However, when these data are plotted against the hydrophobicity descriptor π for the substituent on N (π_N), the values display a linear relationship with $R = -0.90$ (Fig. 6B). Since π describes the hydrophobicity only for the substituent on N and not for the whole molecule, this leads to propose that there exist a local effect on this N moiety, probably associated with the interaction of the molecule with its receptor. This suggests that local effects on specific molecular regions are determinant for molecular interactions and subsequent receptor conformational changes and biological responses. Analysis of RBA showed a similar behavior (Fig. 6C and D). The combination of charge on quaternary ammonium and the length increase on the hydrocarbonated chain favors the affinity for ERα. These similar data for these two biological effects strongly suggest that the decrease on metabolic activity is related to molecular interactions with ERα; however, more data are required to fully demonstrate it.

4. Discussion

Tamoxifen is a selective estrogen receptor modulator widely used in the treatment of estrogen-dependent breast cancer. In order to decrease its non-desirable effects and to elucidate mechanisms of actions, permanently charged tamoxifen derivatives including tamoxifen ethyl bromide, tamoxifen methyl iodide and

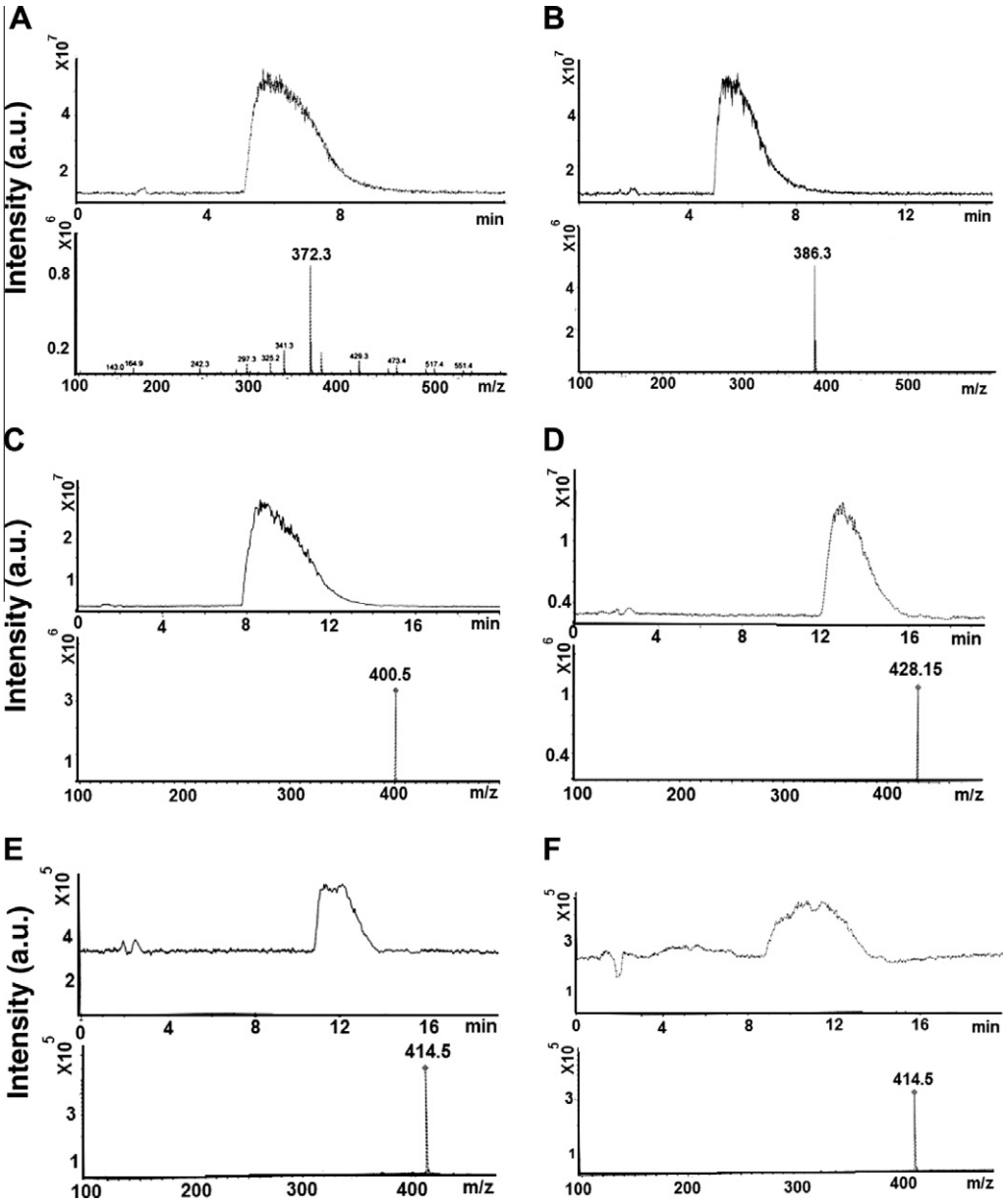


Figure 3. HPLC–MS analysis of PCTDs. The analysis shows the m/z relationships for PCTDs in standard solution. (A) Tamoxifen, (B) tam methyl iodide, (C) tam ethyl bromide, (D) tam butyl bromide, (E) tam isopropyl bromide. The expected m/z values and the absence of a tamoxifen signal in the PCTDs indicate high purity of the compounds. Such purity is also observed for Tam propyl bromide in a cell lysate (F).

tamoxifen butyl bromide, have been produced by several groups.[8,13,14,19–21] Whether or not PCTDs display intracellular and genomic actions remains controversial. Some authors assume that the charged molecules would not enter the cells, which would eliminate the potential genomic effects of PCTDs via interaction with intracellular estrogen receptors. In this manner, either extracellular or non-genomic mechanisms of actions of PCTDs have been proposed to explain the effect of different PCTDs on volume-activated chloride currents in HeLa cells, voltage-gated cationic channels in rat embryonic hypothalamic neurons, apoptosis in acutely damaged mammary epithelial cells, smooth muscle calcium-activated large-conductance potassium channels, and inhibition of intestinal and uterine muscles.[8,13,14,19–21] It has been also suggested that tamoxifen methyl iodide has a poor penetration (5%) lacking in vitro activity.[26] Nevertheless in vivo studies suggest that tamoxifen methyl iodide displays intracellular actions, authors claim that the discrepancies between in vitro and in vivo results may be related to the dynamics of distribution of the compound since in whole animal studies the PCTD was present for longer times.[22,23]

Here we demonstrate that PCTDs can be detected in cell lysates from HeLa cells incubated with the compounds, and that transcriptional activity mediated by ERα is affected by PCTDs. By using mass spectrometry and considering retention times, m/z ratio and fragmentation patterns, we were able to detect and identify all PCTDs in HeLa cell lysates. Such parameters were evaluated in six PCTDs at different incubation times but detectable amounts were achieved only after 48 h of incubation. This observation of the required time to detect PCTDs in cell lysates is in accordance with the in vivo findings which suggest that in whole animal studies the PCTD was present for longer times.[22,23] In the cell lysate analysis, broad peaks were observed in the retention times, which can be explained by the low concentration of the compound present in the lysates, or by additional complex matrix properties from the cell lysates. The differences observed in the fragmentation patterns of Figure 2B and C may be due to the presence of several

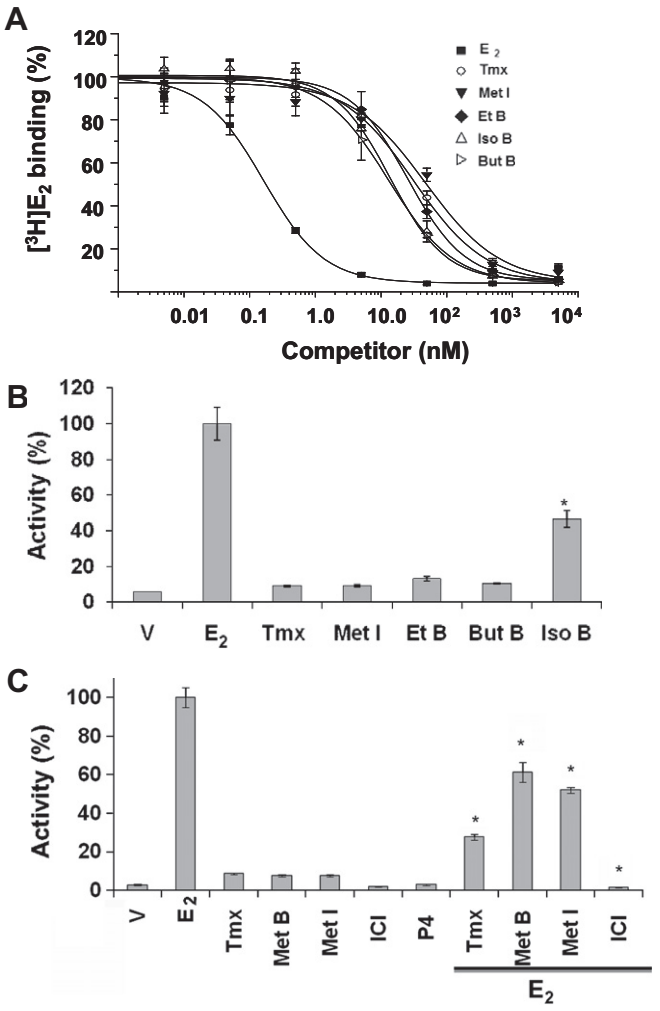


Figure 4. Binding and transcriptional activity of PCTDs. (A) All of the compounds shifted the binding of labeled estradiol in a similar manner to that of tamoxifen. (B) Transcriptional activity of ERα (analyzed by gene reporter assays) was induced by tamoxifen isopropyl bromide in almost 50% of the activity induced by estradiol (normalized to 100%). Neither tamoxifen nor other PCTDs displayed such agonist property. (C) Gene reporter activity induced by estradiol was significantly decreased in the presence of tamoxifen, the antiestrogenic ICI 182, 780 or PCTDs. *V* = vehicle, E_2 = estradiol, *Tmx* = tamoxifen, *Met I* = tamoxifen methyl iodide, *Met B* = tamoxifen methyl bromide, *Et B* = tamoxifen ethyl bromide, *But B* = tamoxifen butyl bromide, *Iso B* = tamoxifen isopropyl bromide, *ICI* = ICI 182, 780, *P4* = progesterone. *Columns*, mean; *bars*, ±s.d. $^*P$ <0.005.

Table 3
Inhibition constant (K_i) and relative binding affinities (RBA) of PCTDs to ERα (obtained from binding experiments)

Compound	K_i (nM)	RBA (%)
E_2	0.16	100
Tamoxifen	31.19	0.51
T. methyl iodide	46.29	0.35
T. ethyl bromide	13.66	1.17
T. isopropyl bromide	25.43	0.63
T. butyl bromide	13.01	1.23

E_2 = estradiol, T = tamoxifen.

components present in very low concentrations in the cell lysates, making difficult to clearly appreciate fragments of the corresponding PCTD.

Fisher et al.[32] estimated permeation properties of several positively charged compounds (from parallel artificial membrane permeability assays) concluding that permanently charged molecules

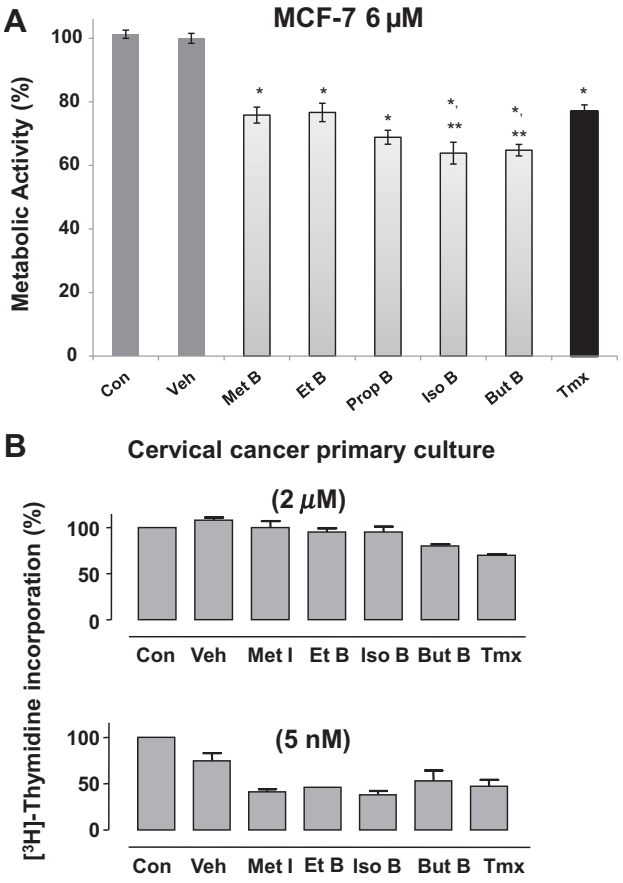


Figure 5. Effect of PCTDs on cancer cell proliferation. (A) All PCTDs significantly decreased proliferation of MCF-7 breast cancer cells (6 μM, 96 h treatment) in comparison to untreated cells (control). Besides tamoxifen isopropyl bromide and tamoxifen butyl bromide show a stronger antiproliferative effect than tamoxifen. (B) ^{3}H thymidine incorporation in cervical cancer cells from a primary culture was not affected by 2 μM PCTDs however, a lower concentration (5 nM) significantly decreased thymidine incorporation. *Con* = control, *Veh* = vehicle, *Met B* = tamoxifen methyl bromide, *Met I* = tamoxifen methyl iodide, *Et B* = tamoxifen ethyl bromide, *Prop B* = tamoxifen propyl bromide, *Iso B* = tamoxifen isopropyl bromide, *But B* = tamoxifen butyl bromide, *Tmx* = tamoxifen. *Columns*, mean; *bars*, ±s.e.m. $^*P$ <0.001 in comparison to vehicle. $^{**}P$ <0.001 in comparison to tamoxifen.

might display high passive diffusion provided that the charge can be spread by several aromatic rings. Actually, they suggest that there is not an apparent association between partition coefficient values (Log *P*) and permeation properties.

Binding studies demonstrated that PCTDs affinity to ERα is similar to that of tamoxifen, as previously reported for tamoxifen methyl iodide and ethyl bromide.[26] Among PCTDs, methyl tamoxifen displayed the lower affinity to ERα while butyl tamoxifen showed the higher. The structure–activity analysis (Fig. 6) suggests that PCTDs permanent charge and the increment on the hydrocarbonated chain, favors the affinity for ERα. Besides, the decrease on metabolic activity of MCF-7 cells might be associated to molecular interactions with ERα. It would be very interesting to synthesize more PCTDs in order to perform a complete QSAR analysis.

Certain key regions of tamoxifen are thought to be important for its binding to ER and to block estrogen action,[33] the amino ethoxy side chain of the α′ ring is critical for its antiestrogenic activity.[34] These findings together with our results suggest that the alkylaminoethoxy chain of PCTDs is an important region both for the activity of the molecule as well as for the affinity to ERα. Of course also the status of the receptor is important in the response to tamoxifen. For example, it has been reported that tamoxifen can

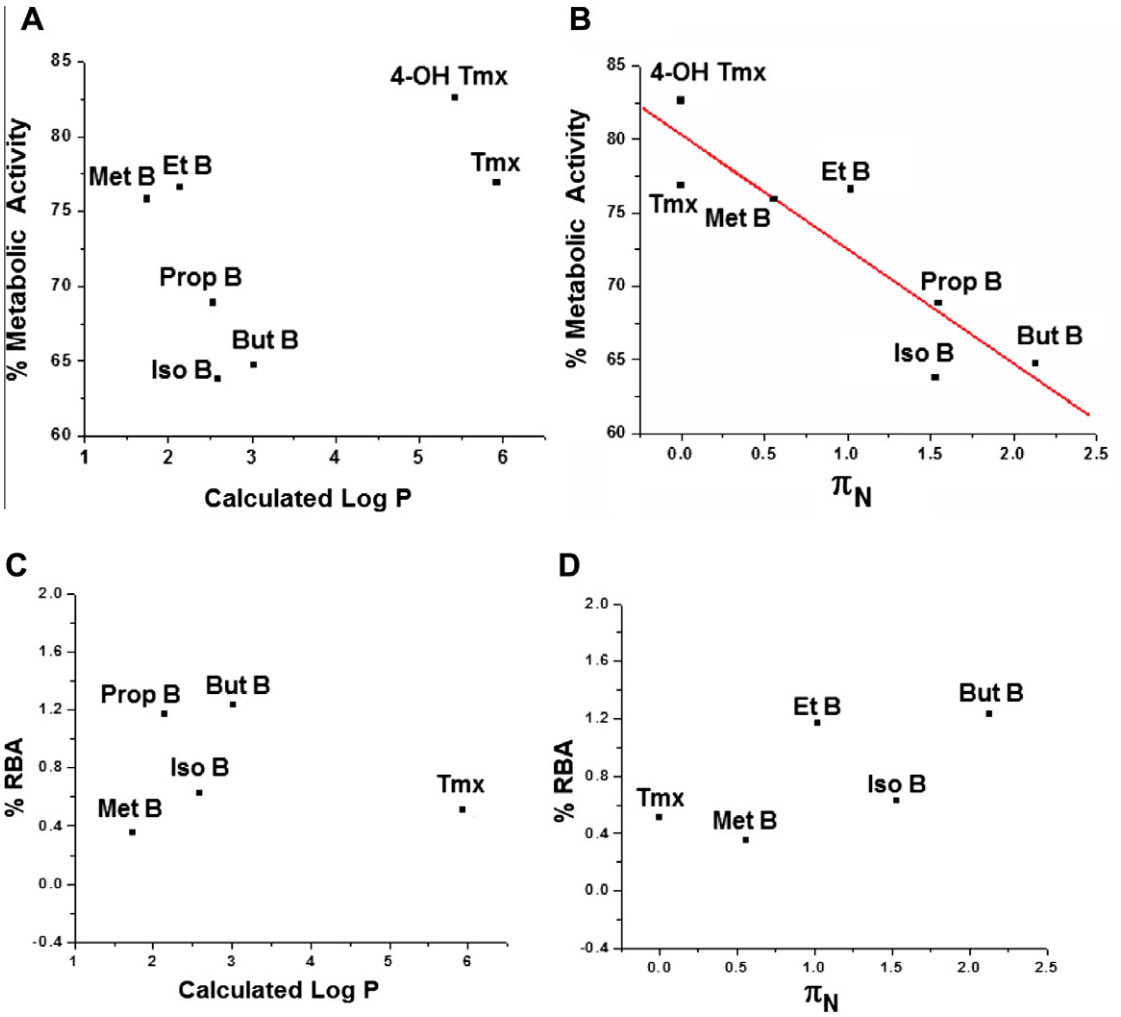


Figure 6. Structure–activity relationship. (A) Partition coefficient calculated with ALOGPS 2.1 versus metabolic activity (%) of MCF-7 cells (from Fig. 4). (B) Hydrophobicity descriptor π for the substituent on N (π_N) versus metabolic activity (%) of MCF-7 cells. (C) Partition coefficient (calculated Log *P*) versus RBA (%) to ERα. (D) Hydrophobicity descriptor π for the substituent on N (π_N) versus RBA (%) to ERα.

switch from ERα-antagonist to ERα-agonist if ERα is phosphorylated by PKA.[35]

Potential genomic action of PCTDs was assessed by ERα-mediated transcription. Interestingly, tamoxifen isopropyl bromide alone induced transcriptional activity, while tamoxifen methyl bromide and tamoxifen methyl iodide significantly decreased the gene reporter activity in the presence of estradiol. Then, substitutions on the nitrogen of a branched chain, switch tamoxifen from an antiestrogen to an estrogen agonist. These gene reporter assays strongly suggest that PCTDs might influence cell physiology by binding to ERα. Since the inhibition constants observed is in the nanomolar range (Table 3), and compounds are tested at micromolar concentrations, then even penetrations below 1% would be needed in order to interfere with the genomic actions of ERα. This scenario could be possible if relatively long incubation times are considered. Actually, detection of PCTDs in cell lysates and effects on transcriptional activity were only observed after incubating the cells with the compounds during 48 h, in contrast to tamoxifen which could be detected in cell lysates after 24 h of incubation (data not shown). The required time to reach relevant concentrations might indeed explain differences between the in vitro and in vivo findings obtained by different researchers. It would be very interesting to quantify the intracellular concentration of PCTDs when incubating the cells at different extracellular concentrations of the compounds and to know whether such intracellular concentration is saturable. It would be also important to study the effect of PCTDs on ERβ.

Finally, we observed that PCTDs decrease proliferation of cancer cells in a cell-type and concentration-dependent manner. PCTDs were tested on the proliferation of MCF-7 at different concentrations (5 nM, 2 μM, and 6 μM) and different incubation times (24, 48 and 96 h). Significant effects were only observed with the treatment at 6 μM and during 96 h. Noteworthy, the effect of propyl tamoxifen and tamoxifen butyl bromide was higher in comparison to that of tamoxifen alone. A higher anticancer activity in vivo was already observed for tamoxifen methiodide (tamoxifen methyl iodide) in comparison to tamoxifen.[22] Interestingly, PCTDs decreased proliferation of cervical cancer cells only at the lowest concentration (5 nM). Differential concentration effects of tamoxifen on cervical cancer cells have been already reported. For example, tamoxifen treatment (5 μM, six day exposure) resulted in 66–74% growth inhibition of cervical cancer cell lines including HeLa cells;[36] but in the cervical cancer cell line SFR lacking ERs, low concentrations of tamoxifen (1×10^{-9} or 1×10^{-11} M, five day treatment) stimulated HPV-16 gene expression and cell proliferation.[37] Clinical studies showed that 16% of cervical cancer biopsies from patients treated with tamoxifen (given orally during 10 days) had a significant decrease in the number of mitotic figures.[38] In summary, the response of cervical cancer cells to tamoxifen is highly variable depending on different aspects including treatment conditions, differences in

HPV copy number, different amounts of ERs and tamoxifen concentration (probably tamoxifen has different targets at higher concentrations avoiding its antiproliferative effects). Thus, it would be very important to test the effects of PCTDs on cell proliferation of different cervical cancer cells, both in cancer cell lines and primary cultures from different patients. Whether or not such variable response would be eliminated by PCTDs remains to be elucidated. It will be also very important to discover additional targets of PCTDs. Actually, the effect of PCTDs on cell proliferation might be also explained by the inhibition of some ion channels for example the *ether à-go-go related gene* potassium channel (Kv11.x), which is known to be inhibited by tamoxifen and to be an advantage for proliferation of several cancer cells.[39,18]

Despite a quantitative study of the intracellular concentration of PCTDs is necessary and the precise mechanism by which PCTDs enter the cells remains elusive, here we show that PCTDs display genomic action by affecting transcriptional activity of ERα and influence cancer cell proliferation. The values obtained (retention time value, *m*/*z* ratio and fragmentation pattern) for the standard solutions using the compounds in the micromolar range, and the agonistic effects of two PCTDs alone on the transcriptional activity show that the compounds are not contaminated with possible unmodified tamoxifen. To our knowledge, three of these PCTDs, namely, tamoxifen isopropyl bromide, tamoxifen methyl bromide and tamoxifen propyl bromide, are new compounds not previously reported.

5. Conclusions

Due to the relevance of tamoxifen in oncology, the necessity to have new anticancer drugs, and in order to gain insights into the mechanisms of action of permanently charged tamoxifen derivatives, we synthesized, characterized, and studied the genomic actions of these compounds including three new derivatives. Our results strongly suggest genomic effects as a mechanism of action of permanently charged tamoxifen derivatives. Detection of compounds in cell lysates and gene reporter studies provide more accurate information on their potential effects and mechanisms of action. This approach could lead to a better design of new therapeutic molecules and help to elucidate molecular mechanisms of new anticancer drugs.

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