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Short Communication

COMPARISON BETWEEN INHIBITION OF PROTEIN KINASE C AND ANTAGONISM OF CALMODULIN BY TAMOXIFEN ANALOGUES

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Abstract—A variety of analogues of tamoxifen were tested for inhibition of protein kinase C (PKC) activity in MCF-7 breast cancer cells. These results were compared with the calmodulin antagonism exhibited by the analogues as measured by inhibition of calmodulin-dependent cyclic AMP phosphodiesterase. The same structural features that enhanced PKC inhibition also led to an increase in calmodulin antagonism, namely 4-iodination and elongation of the basic side-chain. The most potent analogue has a 4-iodine substituent and eight carbon atoms in its basic side-chain with IC_{50} values of 38 μM for PKC inhibition and 0.3 μM for calmodulin antagonism, which compares with 92 and 6.8 μM, respectively, for tamoxifen. Some selectivity was achieved with a ring-fused analogue that retained the potency of tamoxifen as a PKC inhibitor, but lacked calmodulin antagonism.

Key words: anti-oestrogens; triphenylethylenes; cAMP phosphodiesterase; protein kinase C

Tamoxifen is used widely for the treatment of advanced breast cancer [1]. Besides its action as an anti-oestrogen, tamoxifen has effects on other important components of intracellular signalling pathways, such as antagonism of calmodulin action [2–4] and inhibition of the enzyme PKC¶ [5, 6, 7]. In order to examine the significance of these activities to the antitumour action of the drug, we have reported structural requirements necessary for the interaction with calmodulin, using analogues of tamoxifen [8, 9].

Amongst the 4-substituted analogues, the potency as antagonists of the calmodulin-dependent activity of the cAMP-PDE enzyme increased with lipophilicity and the cytotoxicity towards MCF-7 cells correlated with calmodulin antagonism [8], as has been suggested by others [3]. Tamoxifen was demonstrated to inhibit the PKC enzyme from rat and human tissues with IC_{50} values from 30 to 100 μM, [5, 7, 10] while its metabolites, 4-hydroxytamoxifen and *N*-desmethyltamoxifen, gave values of 25 and 8 μM respectively [6]. A report using MCF-7 cells as a source of the enzyme gave IC_{50} values between 50 and 250 μM for tamoxifen depending on the concentration of phosphatidylserine used in the assay [11]. However, the role, if any, of PKC inhibition in the cytotoxicity displayed by anti-oestrogens against MCF-7 cells remains unclear [11, 12].

Therefore, for this present study, we have compared the calmodulin antagonism exhibited by some tamoxifen analogues with their ability to inhibit PKC activity in the cytosolic fraction of MCF-7 cells. These cells have been reported to contain the α-, γ-, δ-, ε- and ζ-isoenzymes of PKC [13] even though their expression differs depending on cell source. We found the wild type MCF-7 cells to contain abundant amounts of PKC-ε with lesser amounts of PKC-α and PKC-ζ [14]. The assay used here to measure PKC activity utilizes TPA as an enzyme activator. TPA does not activate ζ, so the results obtained with the wild type cytosolic fraction are due to the combined activity of the α- and ε-isoenzymes. The Adriamycin-resistant MCF-7 cells have a different isoenzyme profile with overexpression of the α-isoenzyme [15]. Therefore, the differential expression of PKC-α and PKC-ε in the two cell types would permit detection of any selectivity between the two isoenzymes in susceptibility towards inhibition by the tamoxifen analogues.

Materials and Methods

Chemicals. Tamoxifen was obtained from Aldrich Chemical Co. (U.K.). The analogues were synthesized by the following literature procedures: (**2**) ref. [16]; (**3**) and (**4**) ref. [17]; (**5**), (**6**) and (**7**) ref. [19] and (**10**) ref. [20]. The structures are shown in Fig. 1. The PKC assay kit and [8-^{3}H]cyclic AMP (26 Ci/mmol) were purchased from Amersham International plc (Amersham, U.K.). All other chemicals were purchased from Sigma (Poole, U.K.). Stock solutions of all compounds were prepared in DMSO and stored at −20°.

Determination of PKC activity and calmodulin antagonism. The culture conditions for the wild type and adriamycin-resistant MCF-7 cells were as described [14]. Cells were cultured in Petri dishes (140 mm diameter) and the cytosolic fraction was prepared when they approached confluence. Briefly, plates were placed on ice and washed three times with 10 mL of ice-cold wash buffer (20 mM Tris-HCl, 150 mM glucose, 2 μg/mL leupeptin, 2 μg/mL aprotinin, adjusted to pH 7.4). Cells were scraped into 0.25 mL of ice-cold H8 buffer (20 mM Tris-HCl, 2 mM

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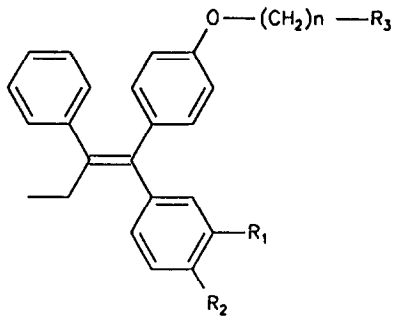
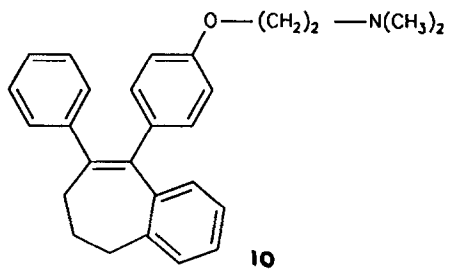
¶ Abbreviations: PKC, protein kinase C; cAMP-PDE, cyclic adenosine monophosphate phosphodiesterase





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Compound	n	R_1	R_2	R_3
tamoxifen **1**	2	H	H	$N(CH_3)_2$
droloxifene **2**	2	OH	H	$N(CH_3)_2$
idoxifene **3**	2	H	I	$N(CH_2)_4$
4	2	H	I	$N(CH_3)_2$
5	4	H	I	$N(CH_3)_2$
6	8	H	I	$N(CH_3)_2$
7	2	H	-C≡CH	$N(CH_3)_2$
8	2	H	H	-N NH
9	2	H	H	$NHCH_3$

Fig. 1. Structures of tamoxifen analogues.

EDTA, 2 mM EGTA, 6 mM β-mercaptoethanol, 2 μg/mL leupeptin, 2 μM/mL aprotinin adjusted to pH 7.5) and were disrupted by sonication using a Branson sonifier 250. The cytosolic fraction was separated from the particulate fraction by ultracentrifugation (100 000 *g* for 30 min, 4°). The cytosolic fraction was diluted by a factor of 5–15 using H8 buffer in order to dilute out endogenous peptide inhibitors which interfere with maximal PKC activity as described in the protocol for the Amersham International assay kit. Aliquots (25 μL) of the dilutions were placed in microplate wells and PKC activity was measured by the incorporation of the γ-phosphate moiety of [^{32}P]ATP into a PKC specific peptide. The kit contains TPA as enzyme activator at a final concentration of 13 μM. Tamoxifen and its analogues at various concentrations were added to aliquots of the cytosolic fraction and the degree of inhibition of PKC activity was calculated as a percentage of enzyme activity in drug-free incubates. Calmodulin-dependent cyclic AMP phosphodiesterase was assayed as previously described [8, 21]. The enzyme was measured using [8-^{3}H] cyclic AMP as substrate and 5′-nucleotidase from snake venom to convert tritiated AMP into adenosine. Batch elution with Dowex anion exchange resin enabled the product nucleosides to be separated from the substrate. The basal activity (calmodulin independent) of the phosphodiesterase was determined by adding 1 mM EGTA to the assay medium.

Results and Discussion

The IC_{50} values for inhibition by the anti-oestrogens of the PKC activity in wild type MCF-7 cells are shown in Table 1. The cytosol of these cells contains a combination of the α- and ε-isoenzymes. Compound **1–4** and **10** were also assayed against the PKC activity, which consists predominantly of the α-isoenzyme, in the cytosol of Adriamycin-resistant MCF-7 cells. These compounds produced IC_{50} values similar in potency to those observed with the wild type cells (data not shown). Therefore, these results are consistent with the notion that the anti-oestrogens lack selectivity towards the various isoforms as has been demonstrated previously [22]. For convenience, the other derivatives were assayed against PKC activity of the wild type cells only. Tamoxifen, **1**, inhibited PKC with an IC_{50} value of 92 μM which is in good agreement with published values [5, 7]. Its major metabolite, *N*-desmethyltamoxifen, with an IC_{50} of 45 μM is a more potent inhibitor as demonstrated by others [6]. This may be due to the *N*-desmethyl group acting as a hydrogen bond donor, which can not occur with the dimethyl substituent. Support for this comes from the increased potency towards PKC exhibited by piperazinotamoxifen, **8**, another analogue with a N–H bond suitable for hydrogen bonding. It is doubtful that the concentrations of tamoxifen and its metabolites attained *in vivo* are sufficiently high to suppress

Table 1. Inhibition of PKC and calmodulin antagonism by tamoxifen analogues*

Compound	PKC inhibition wild type MCF-7 (IC_{50} μM)	Calmodulin antagonism inhibition of cAMP-PDE (IC_{50} μM)†
1	92.3 ± 6.7	6.8 ± 1.0‡
2	95.3 ± 4.2	12.9 ± 2.6
3	70.7 ± 7.1	1.5 ± 0.2
4	48.3 ± 8.3	2.3 ± 0.7‡
5	38.8 ± 1.4	1.3 ± 0.7
6	38.0 ± 1.5	0.3 ± 0.2
7	74.0 ± 14.4	4.6 ± 2.0
8	52.0 ± 5.6	> 50‡
9	45.7 ± 3.8	4.9 ± 1.0
10	90.0 ± 3.0	> 50§

* Each IC_{50} value is the mean of three experiments ±SD.
† None of the compounds inhibited the basal activity of cAMP-PDE when assayed at final concentrations of 10 and 20 μM.
‡ IC_{50} values as reported in ref. [8].
§ IC_{50} value as reported in ref. [29].

PKC activity markedly. In patients, serum concentrations of tamoxifen and *N*-desmethyltamoxifen are around 0.5 μM and intratumoural levels were shown to be 5–10 times higher [23–25]. The lipophilic nature of these compounds may enable them to accumulate within cellular compartments and reach higher concentrations. Droloxifene (3-hydroxytamoxifen) and idoxifene(pyrrolidino-4-iodotamoxifen) are two anti-oestrogens that are in clinical trials [26, 27] and have similar activity against PKC in MCF-7 cells as tamoxifen. However, 4-iodotamoxifen (**4**) showed more potent inhibition with an IC_{50} of 48 μM. A further increase in potency was produced by elongation of the basic side-chain to give the four and eight carbon derivatives, **5** and **6**. Therefore, increasing the lipophilic nature of these anti-oestrogens increases the inhibitory activity towards PKC. This supports the observations that tamoxifen analogues inhibit PKC via interactions with phospholipids [5, 7, 12] which, in turn, blocks the membrane binding of these enzymes [28].

Interestingly, some of the structural features of these compounds which increase inhibition of PKC also attenuate their antagonism of calmodulin function. The 4-iodinated derivative **4**, was approximately three-fold more potent a calmodulin antagonist than tamoxifen and extension of the basic side-chain to give **5** and **6** also produces increases in potency. Indeed, it has been suggested that because tamoxifen as well as other calmodulin antagonists, such as chlorpromazine and trifluoperazine, inhibit PKC activity there must be some homology between the regulatory domain of PKC and calmodulin [5]. However, within this series of triphenylethylene anti-oestrogens we can define the structural features that confer selectivity for calmodulin antagonism. For example, piperazinotamoxifen, **8**, has increased potency as a PKC inhibitor compared with tamoxifen, but it has lost the antagonism of calmodulin function, with no inhibition observed up to a concentration of 50 μM. In addition, replacement of the dimethylamino group, **4**, by pyrrolidino, **3**, caused a modest increase in calmodulin antagonism, but decreased PKC inhibition. These results show that the basicity of the side-chain substituent of tamoxifen is essential for calmodulin antagonism. This is supported by studies undertaken by Bouhoute and Leclercq who demonstrated that the basic side-chain is directly involved in the antagonistic activity of tamoxifen towards the association of the oestrogen receptor with calmodulin coupled to Sepharose [4]. Compound **10**, which has the same RBA to the oestrogen receptor as tamoxifen [29], has a similar potency as a PKC inhibitor, but lacks the calmodulin antagonism. Its structure differs from tamoxifen only in that the ethyl group is constrained into a fused ring conformation [30]. Therefore, a study of the properties of this ring-fused analogue and related derivatives might shed further light on the mechanism of action of tamoxifen as a chemotherapeutic agent.

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