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VARIATION OF THE INHIBITION OF CALMODULIN DEPENDENT CYCLIC AMP PHOSPHODIESTERASE AMONGST ANALOGUES OF TAMOXIFEN; CORRELATIONS WITH CYTOTOXICITY

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Abstract—The ability of a variety of analogues of tamoxifen to inhibit calmodulin dependent cyclic AMP phosphodiesterase has been determined. Effective inhibition requires that the aminoethoxy side chain bears a positive charge at physiological pH and is not too bulky. Amongst 4-substituents, inhibitory potency increases with lipophilicity. The stereochemistry about the olefinic linkage is not important. The most potent agent found (IC_{50} 1.4 μM, compare tamoxifen = 6.75 μM) has a 4-iodine substituent and pyrrolidino in place of dimethylamino. This analogue is also more cytotoxic than tamoxifen against MCF-7 human breast cancer cells as determined in a 24-hr assay, but there was no correlation found between calmodulin inhibition and cytotoxicity against the L1210 murine leukaemia or Walker rat carcinosarcoma cells in culture. The results are consistent with the possibility that calmodulin is important to the functioning of oestrogen receptor mediated growth in MCF-7 cells.

Calmodulin plays a key role in processes that govern cell proliferation [1, 2] and consequently the pharmacological effects of inhibitors of its function [3, 4] are of considerable interest. Since the discovery by Lam [5] that the antioestrogen tamoxifen (**1**) is a competitive inhibitor of the activation of cyclic AMP phosphodiesterase (cAMP-PDE) by calmodulin, action on calmodulin has been suggested to contribute to the inhibition by tamoxifen of hormone responsive breast cancer cell growth [6, 7]. Indeed, there is a component of the inhibitory action of tamoxifen not reversible by the addition of oestrogen [8] which has been shown to correlate with the calmodulin inhibition [9], and a similar correlation has also been noted for the antioestrogen nitromifene and one of its metabolites [10]. The inhibition of cAMP-PDE in particular may be of relevance since the ability of tamoxifen to antagonize the trophic effects of oestrogen on the quail oviduct has been attributed to the regulation of cAMP levels [11].

Studies of the structure–activity relationships against calmodulin amongst tamoxifen derivatives have so far been restricted to tamoxifen and its metabolites [7, 12]. Our synthetic program has provided us with a considerable range of analogues of tamoxifen for which oestrogen receptor binding and inhibition of proliferation of the MCF-7 human breast cancer cell line have been reported [13]. We report here the varying ability of these compounds to inhibit the calmodulin dependent cAMP phosphodiesterase. In addition, we show that for selected analogues, there is a correlation between calmodulin inhibition and cytotoxicity against MCF-7 cells, but not against L1210 or Walker cells in culture.

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MATERIALS AND METHODS

Analogues of tamoxifen were synthesized by the following literature procedures (see Table 1 for structures): (**1**) Ref. 14; (**2**), (**3**), (**16**), Ref. 15; (**5**) Ref. 16; (**6**), Ref. 17; (**7**), Ref. 18; (**8**)–(**10**) and (**12**)–(**14**), Ref. 13; and (**11**), Ref. 19. Compound (**4**) not previously described was prepared from (**3**) by condensation with butyroyl chloride (2 equiv.) in dichloromethane and the amide (87% yield), m.p. 94–95° (Anal. C, 79.5; H, 8.1; N, 5.8; $C_{32}H_{38}N_2O_2$ requires C, 79.6; H, 7.9; N, 5.8) reduced with excess lithium aluminium hydride in tetrahydrofuran at reflux for 2 hr to give (**4**) (75% yield), m.p. 86–87° (Anal. C, 82.0; H, 8.85; N, 5.9, $C_{32}H_{40}N_2O$ requires C, 82.0; H, 5.6; N, 6.0%).

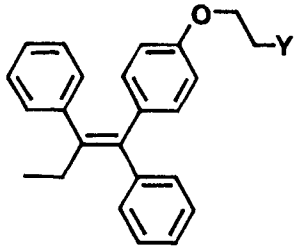
Inhibition of calmodulin dependent cAMP-PDE

Materials. cAMP, pure pig brain calmodulin and calmodulin deficient phosphodiesterase (from beef heart) were obtained from Boehringer Mannheim (Lewes, U.K.). [^{3}H]cAMP was obtained from Amersham International (Bucks, U.K.). Dowex 1-X8 (200–400 mesh) anion exchange resin and snake venom (*Ophiophagus hannah*) were purchased from the Sigma Chemical Co. (Poole, U.K.).

Method. The calmodulin dependent cAMP-PDE activity was assayed with [2,8-^{3}H]cyclic-3:5-AMP as substrate for the cAMP-PDE as described by Thompson *et al.* [20] and MacNeil *et al.* [21]. Purity of the [^{3}H]cAMP was checked before use by thin layer chromatography on silica gel plates using *n* propanol:ammonia:water (6:3:1) as solvent system. Optimal assay conditions consisted of 40 mM Tris–HCl pH 7.4, 4 mM dithiothreitol, 5 mM $MgCl_2$, 100 μM cAMP and [^{3}H]cAMP (2×10^5 cpm per tube). The basal activity was determined by adding

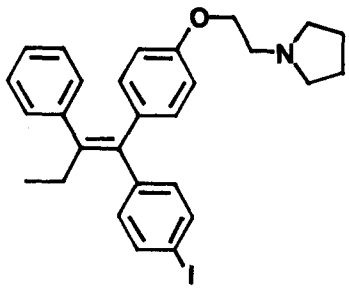
Table 1. Inhibition of calmodulin dependent cAMP-PDE by tamoxifen and various analogues: structures and IC_{50} values (± SD for triplicate determination)

Variation of side chain



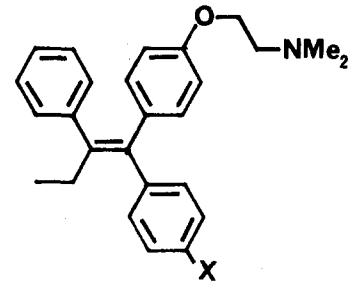
(1) Y = NMe_2 (tamoxifen) 6.75 ± 1.06 μM
(2) Y = NEt_2 10.0 ± 1.2 μM
(3) Y =  >50 μM
(4) Y =  >50 μM
(5) Y = $\overset{+}{N}Me_2—O^-$ >50 μM
(6) Y = $\overset{+}{N}(Et)Me_2\ Br^-$ 4.75 ± 1.06 μM
(7) Y = $N(Me)CH_2CF_3$ >50 μM

Other

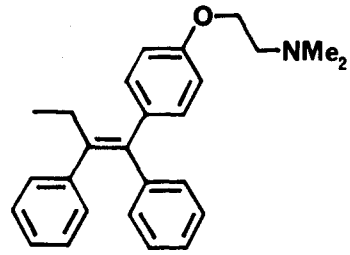


(15) 1.45 ± 0.08 μM

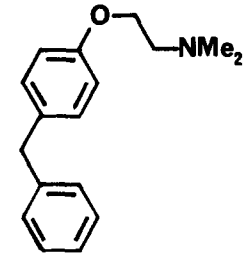
Variation of 4-substituent



(8) X = —Cl 4.8 ± 0.24 μM
(9) X = —Br 2.0 ± 0.22 μM
(10) X = —I 2.3 ± 0.42 μM
(11) X = —OH 19.0 ± 2.85 μM
(12) X = —CHO >50 μM
(13) X = $—CH_2OH$ >50 μM
(14) X =  6.0 ± 0.54 μM



(16) *cis*-tamoxifen 6.5 ± 0.52 μM



(17) >50 μM

1 mM EGTA to the incubation medium. For calmodulin dependent activity the assays were supplemented with 25 μM $CaCl_2$ and various concentrations of calmodulin. The calmodulin independent activity was assayed in the absence of calmodulin. The final assay volume was 800 μL and each sample was run in duplicate. Incubations at 37° were started by the addition of the cAMP-PDE enzyme. Aliquots (400 μL) were withdrawn after 10 and 20 min of incubation, to check the linearity of the reaction, and terminated by placing in a boiling water bath for 45 sec. The tritiated AMP formed during the incubation was then converted into tritiated adenosine by the 5'-nucleotidase in snake venom. Product nucleosides were separated from unreacted substrate using Dowex anion exchange resin and the tritiated adenosine determined by liquid scintillation counting. The incubation time and enzyme concentration was now adjusted so that substrate hydrolysis did not exceed 25%. The assays were carried out in the presence and absence of different concentrations of the triphenylethylene derivatives dissolved in methanol to give a final concentration of 1.5% in the assay mixture. At this concentration methanol had a small inhibitory effect (10%) on the calmodulin dependent cAMP-PDE activity. The results are expressed as the concentration of inhibitor giving 50% inhibition of the calmodulin dependent cAMP phosphodiesterase activity (IC_{50}).

Cytotoxicity against MCF-7 cells

MCF-7 cells were originally obtained from Michigan Cancer Foundation (Detroit, MI). They were maintained in routine culture in Dulbecco's modified Eagles medium (DMEM) supplemented with L-glutamine (2 mM) gentamycin (1 mg/mL), fungisone (2.5 μg/mL), insulin (10 μg/mL) and 5% foetal bovine serum (FBS).

Cytotoxicity test. MCF-7 cells were plated in 96-multiwell plates, 5×10^3 cells/well in 200 μL DMEM containing 2% carbon stripped FBS [22]. After 24 hr at 37° in a humidified atmosphere the various drugs were added in fresh medium and the plates incubated

for a further 24 hr. Control wells not containing drugs were treated similarly. To estimate the number of live cells present after 24 hr drug contact, the MTT test, originally developed by Mossman [23] and later modified by Twentyman and Luscombe [24] was employed. Briefly, the water-soluble dye MTT, 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co.) is added, 50 μL/well of 5 mg MTT/mL of phosphate buffered saline and the plates incubated a further 4 hr. After removal of the medium, the water-insoluble dye formazan (formed from MTT by action of various dehydrogenases present in the live cells) is dissolved in dimethyl sulphoxide (DMSO) (200 μL/well), placed on a plate shaker for 10 min and optical densities read immediately at 540 nm on a multiscan spectrophotometer. Triplicate wells were analysed for each drug concentration. Preliminary studies (not shown) indicated that the MTT conversion rate increased linearly with cell number over a range of cell concentrations.

Cytotoxicity against tissue culture cell lines L1210 murine leukaemia and Walker rat carcinosarcoma

The L1210 and Walker are tissue culture lines which are used for *in vitro* screening in the Drug Development Section of the Institute of Cancer Research. The L1210 is grown in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and glutamine. The Walker is cultured in DMEM supplemented with heat-inactivated horse serum together with glutamine and is grown in a gassing incubator in 5% CO_2/95% air.

Dose–response experiments were set up in duplicate at a cell density of 1.5×10^4/mL. Cell counts were measured in a Coulter Electronics particle counter and the percentage inhibition of growth was determined after 48 hr of continuous exposure to the drug at 10^{-4}, 10^{-5} and 10^{-6}M.

All analogues of tamoxifen were dissolved in DMSO so that the final concentration during the 48-hr exposure was 0.5%.

RESULTS

Inhibition of calmodulin dependent cAMP-PDE

In all the enzyme assays, product formation was linear up to 20 min of incubation. Saturating conditions were achieved with 100 ng (3.6 units) of calmodulin giving a maximum velocity to the calmodulin dependent activity of 3.13 nmol of cAMP hydrolysed/min/mg. The basal phosphodiesterase activity, with 1 mM EGTA, was 0.46 nmol/min/mg and was identical to the calmodulin independent activity. Table 1 shows the activity of various triphenylethylene derivatives towards the calmodulin dependent enzyme activity. The dose–response curves for compounds (**1**), (**10**), (**11**) and (**15**) are shown in Fig. 1. 4-Hydroxytamoxifen (**11**) has been previously reported to be less potent than tamoxifen against calmodulin [11]. None of the compounds in Table 1 gave any significant inhibition of the calmodulin independent cAMP-PDE activity when assayed at final concentrations of 10 and 20 μM. In addition, compounds (**1**), (**8**), (**10**), (**11**) and (**15**) were tested against the basal cAMP-PDE assay in the presence of 1 mM EGTA. None of these analogues gave significant activity when tested at 10 and 20 μM. These results demonstrate that the tamoxifen derivatives only inhibit the Ca^{2+}/calmodulin stimulated cAMP phosphodiesterase activity and have no direct effects on the enzyme itself.

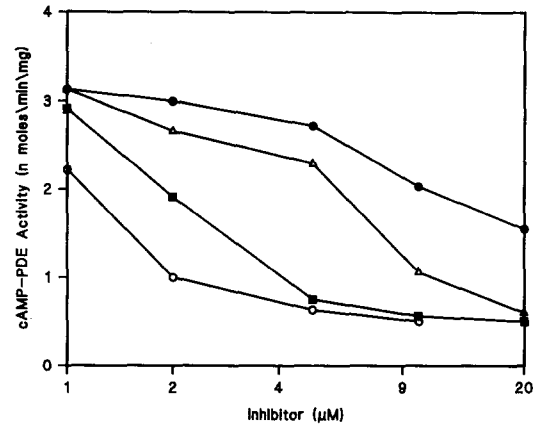


Fig. 1. Inhibition of calmodulin stimulated phosphodiesterase activity by tamoxifen (△), 4-hydroxytamoxifen (●), 4-iodotamoxifen (■) and pyrrolidino-4-iodotamoxifen (○). The assays were carried out as described in Materials and Methods with 100 ng (3.6 units) of calmodulin. Enzyme activity in the absence of inhibitor and presence of calmodulin was 3.13 nmol cAMP hydrolysed/min/mg, while basal activity was 0.46 nmol/min/mg. Each point is the mean of three determinations, which were ± 10%.

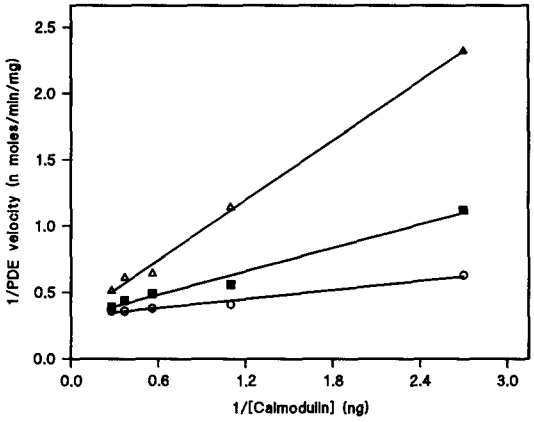


Fig. 2. Double reciprocal plot of the inhibition of calmodulin stimulated phosphodiesterase activity by pyrrolidino-4-iodotamoxifen at 1 μM (■) and 2 μM (△). Control values (○) contained 1.5% methanol. Each point represents the mean (± 10%) of triplicate determinations.

A detailed kinetic analysis of the inhibition of Ca^{2+}/calmodulin dependent cAMP-PDE by pyrrolidino-4-iodotamoxifen (**15**) is shown in the Lineweaver–Burk plot (Fig. 2). Extrapolation of the graphical data produces a common intercept on the vertical axis suggesting that (**15**) is a competitive inhibitor. The K_i value was calculated to be 0.33 μM. Tamoxifen has been shown to be a competitive

Table 2. Cytotoxicity against MCF-7 cells

Compound	% Absorbance relative to control at 2 μM	5 μM	10 μM	20 μM	IC_{50}/μM* ± SD
(1) (tamoxifen)	91	117	104	1	15.9 ± 0.6
(1) + 10^{-8}M E_2	79	120	118	0	
(11) (4-hydroxytamoxifen)	136	158	135	3	15.5 ± 0.7
(11) + 10^{-8}M E_2	114	133	129	0	
(10) (4-iodotamoxifen)	116	135	29	4	7.63 ± 0.06
(10) + 10^{-8}M E_2	110	132	14	0	
(15)	106	119	29	3	8.5 ± 0.2
(15) + 10^{-8}M E_2	102	124	6	0	

* IC_{50} values were determined in a separate experiment using triplicate samples at a drug concentration either side of the IC_{50} value and assuming a linear dose–response between these points. These concentrations are: (1) and (11), 10 and 20 μM; (10), 7 and 8 μM; (15), 8 and 9 μM.

inhibitor of the calmodulin stimulated cAMP-PDE [5, 10] and previous workers, using the beef heart cAMP-PDE enzyme obtained a K_i value of 0.96 μM [5].

Variation of the side chain revealed strict requirements for activity. Replacement of dimethylamino by diethylamino reduced activity and introduction of a piperazino group abolished it. The lack of activity of the trifluorethyl analogue (7) can be attributed to the low basicity of its nitrogen atom. The trifluoromethyl group is known to decrease the basicity by 4 pH units [25] so that unlike the amines (1)–(4), (7) will not be protonated at physiological pH. It would therefore seem that tamoxifen binds to calmodulin in its protonated form. Indeed, for trifluoroperazine, it has been suggested that it is the protonated drug that interacts in an ionic association with calmodulin deprotonated on one of its acidic residues [26]. Further evidence for such an electrostatic interaction in the case of tamoxifen binding is the good activity of the quaternized derivative (6) which is constrained to be positively charged contrasting the inactive *N*-oxide (5) where, although the nitrogen atom bears some degree of positive charge, the molecule as a whole is constrained to be neutral.

Variation of the 4-substituent also profoundly influenced the inhibitory potency and there was a correlation with the lipophilicity of this substituent. Pi-values of hydrophobicity [27] for the substituents are in the order: $—CH_2OH = -1.03$, $—OH = -0.67$, $—CHO = -0.65$, $—H = 0$, $—Cl = +0.71$, $—Br = +0.86$, $—I = +1.12$, which is the order of increasing calmodulin inhibition apart from bromine and iodine substituents which give approximately equal potency and the aldehyde which is less potent than 4-hydroxytamoxifen. Therefore the 4-substituent will rest in a hydrophobic domain in the calmodulin protein. The epoxide (14) was assayed because of the possibility that it could alkylate calmodulin to give irreversible binding, but this was not the case since the IC_{50} value correlated well with the polarity of this substituent.

The most potent triarylethylene derivative so far found against the calmodulin activation of cAMP-PDE was the analogue (15) ($IC_{50} = 1.4$ μM) of 4-iodotamoxifen having a pyrrolidino substituent in place of dimethylamino. In comparison the imidazole based potent calmodulin inhibitor calmidazolium (substance R24571) gave in our assay an IC_{50} value of 0.3 (± 0.05) μM. The stereochemistry about the olefinic linkage was not important since *cis*-tamoxifen had a practically identical potency to the *trans*-isomer. However, the part of the molecule containing the phenyl and ethyl groups is necessary for activity since the diphenylmethane derivative (17) did not inhibit calmodulin. The compound homologous to (17) having diethylamino instead of dimethylamino has been extensively studied by Brandes *et al.* [28] and consistent with our results is reported to inhibit calmodulin dependent mysoin light chain kinase only at very high concentrations (> 1 mM).

Cytotoxicity against the MCF-7 human breast cancer cell line

Four compounds were chosen for study of the cytotoxicity against the MCF-7 cell line. These were tamoxifen, 4-hydroxytamoxifen which is a potent antioestrogen but gives reduced calmodulin inhibition, and the 4-iodinated analogues (10) and (15) which are potent inhibitors of calmodulin as well as being reported to be more effective than tamoxifen at inhibiting the proliferation of MCF-7 cells in culture at 10^{-8} to 10^{-6} M in a five-day assay [13]. The assay reported herein was carried out over a time period of only 24 hr and the results obtained are presented in Table 2. This appears to be an insufficient time period to allow the development of the antiproliferative action that correlates with oestrogen receptor binding. Studies of cytotoxicity against the MCF-7 cell line reported by Murphy and Sutherland [8] alternatively employed an amount of oestradiol (10^{-6} M) that saturates the oestrogen receptors. That our assay conditions give a measure of cytotoxicity is evident from: (i) the high concentrations of drug required; (ii) the absence of

Table 3. Cytotoxicity against L1210 murine leukaemia cells and Walker rat carcinosarcoma cells in culture

	% Inhibition of growth†					
	L1210			Walker		
Compound*	10^{-4}M	10^{-5}M	10^{-6}M	10^{-4}M	10^{-5}M	10^{-6}M
(7)	45	8	3	67	12	15
(17)	76	22	9	89	18	1.3
(2)	92	31	23	99	10	0
(1)	84	13	21	91	18	7
(14)	91	36	2	100	11	15
(10)	95	21	15	100	0	0
(15)	92	29	10	100	0	0

* Compounds are listed in order of increasing calmodulin inhibitory potency.
† Determined after 48 hr of continuous drug exposure (mean of duplicate determinations).

correlation with binding affinity values; and (iii) the sudden onset of cytotoxicity with rising concentration which is similarly observed by Murphy and Sutherland [8]. At the lower concentrations of drug (2–5 μM), the compounds gave some stimulation of growth which might be due to their oestrogen agonist activity. On the other hand, at 20 μM all of the compounds were completely cytotoxic. Differences between the compounds are best observed at 10 μM and the order of increasing cytotoxicity is 4-hydroxytamoxifen (**11**) < tamoxifen (**1**) $\ll$ (**10**) $\simeq$ (**15**). Thus, 4-hydroxytamoxifen, despite its 100-fold greater binding affinity for the oestrogen receptor is the least cytotoxic of the compounds studied. This order of cytotoxicity essentially correlates with the calmodulin inhibition. The presence of oestradiol at 10^{-8} M did not greatly affect the cytotoxicity of the compounds though it seemed to slightly enhance that of the iodinated analogues (**10**) and (**15**). A subsequent experiment using a narrower concentration range to determine the IC_{50} values did not so clearly reveal the correlation with calmodulin inhibition but did show that the presence of the 4-iodine substituent causes a halving of the IC_{50} value.

Cytotoxicity against the L1210 murine leukaemia and Walker rat carcinosarcoma cell lines

A selection of the tamoxifen analogues were tested for inhibition of growth of the L1210 and Walker cell lines in order to determine whether the inhibition of calmodulin correlates with an unselective cytotoxic action. The percentage inhibition of growth at drug concentrations of 1, 10 and 100 μM are given in Table 3. The diphenylmethane derivative (**17**) which did not inhibit calmodulin was as effective at inhibiting these cell lines as the triphenylethylene derivatives. This result is in keeping with the observations of Brandes *et al.* [29] on its diethylamino homologue which was found to have antiproliferative activity that correlated with antagonism of a novel histamine type receptor. There was clearly no correlation with the calmodulin inhibition and the criterion for cytotoxicity seems to be simply the presence of a basic side chain since compound (**7**), in which basicity of the nitrogen is much reduced by the fluorine atoms, was the least inhibitory against the L1210 line and relatively weakly inhibitory against the Walker cell line. The lack of a correlation is in apparent contrast to the observations of Hait and DeRosa [3, 4] who have suggested that the cytotoxicity against L1210 cells correlates with the calmodulin inhibition. However, this correlation is based on results from compounds having a broad range of inhibitory potencies. There was no such correlation when comparing trifluoroperazine, fluphenazine, and chloropromazine [4] so it is likely that factors other than the calmodulin inhibition affect the cytotoxicity towards this cell line.

DISCUSSION

The structure–activity relationships for inhibition of calmodulin activation of cAMP phosphodiesterase upon varying the alkylaminoethoxy side chain lead to the inference that tamoxifen is in its protonated form when it binds to calmodulin. On the other hand, previous experiments have shown that quaternized tamoxifen derivatives do not inhibit MCF-7 cell growth attributed to the inability of these charged species to cross the cell membrane [17]. Therefore, it is the ability of tamoxifen to interchange between protonated and neutral forms that will enable it to inhibit calmodulin in intact cells. The structure–activity studies also indicate that apart from an electrostatic interaction to calmodulin involving the basic side chain, hydrophobic interactions to the triphenylbutene framework are important for binding, and in particular, adding a hydrophobic 4-substituent increased inhibitory activity.

The best inhibitory activity towards calmodulin was found in the pyrrolidino analogue (**15**) of 4-iodotamoxifen, a competitive inhibitor of K_i = 0.33 μM and which is only 4–5-fold less potent than calmidazolium in our assay. It may be possible to exploit the triphenylbutene nucleus further in the design of still more potent calmodulin inhibitors. Nonetheless the iodotamoxifens uniquely combine both improved oestrogen receptor binding with improved calmodulin inhibition compared with tamoxifen. It is possibly this combination of features that causes the iodotamoxifen derivatives to be so much more effective than tamoxifen in their reported

antiproliferative action against the MCF-7 breast cancer cell line in culture [13]. The analogue (**15**) in particular should be of further use in determining the biological consequences of combining good calmodulin inhibition with binding for the oestrogen receptor.

The results of the determinations of cytotoxicity towards MCF-7 cells support entirely the studies of Sutherland *et al.* [9] that the oestrogen irreversible component of the growth inhibitory action of tamoxifen correlates with calmodulin inhibition. Also, consistent with this proposal, is the literature report that the calmodulin inhibitor W13 is completely toxic (99% cell death) to Chinese hamster cells at 40 μg/mL but non-cytotoxic at 30 μg/mL [1]. This narrow concentration range, over which the drug exerts its effect and which may be a feature of calmodulin inhibition parallels the observed effect of the tamoxifen derivatives against MCF-7 cells.

Most importantly the calmodulin inhibition does not correlate with cytotoxicity towards the L1210 and Walker cell lines so it is a reasonable presumption that there is coupling between the oestrogen receptor and calmodulin in the pathways that control the rate of proliferation of hormone responsive breast cancer cells. Such a possibility has been already raised following comparisons of the actions of antioestrogens and calmidazolium on the MCF-7 cell cycle where both types of compound block the cycle at the same point [30]. This may be associated with the reported finding that a tyrosine kinase which may activate the oestrogen receptor is calmodulin dependent [31]. Another possibility is that the hormone receptors could transport the drugs to the appropriate cellular site [32] where calmodulin is important.

Our results indicate that combining a good calmodulin inhibitor with a compound that binds to the oestrogen receptor might provide a strategy that could ultimately yield new drugs for the treatment of hormone responsive breast cancer.

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