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Preclinical report

New calmodulin antagonists inhibit *in vitro* growth of human breast cancer cell lines independent of their estrogen receptor status

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Calmodulin plays a key role in the regulation of cell proliferation and calmodulin antagonists may offer a new therapeutic approach in the treatment of breast cancer. Three new specific calmodulin antagonists with improved potency were synthesized and screened on human breast cancer cell lines known to be estrogen receptor (ER)-positive or -negative. These calmodulin antagonists significantly inhibited cell growth as measured by the MTT proliferation assay ($p < 0.001$). Their IC_{50} values were in the low micromolar range against both ER-positive and -negative variants of the MCF-7 cell line. Two other breast cancer cell lines (ER-positive T-47D and ER-negative MDA-MB-231) were also inhibited by these calmodulin antagonists with IC_{50} values in a similar range. The level of inhibition was independent of any stimulation of cell growth by estradiol. Calmodulin antagonists effectively reduced cell growth of both ER-positive and -negative human breast cancer cell lines *in vitro*. Calmodulin antagonists represent a novel therapeutic approach requiring further investigation. [© 2000 Lippincott Williams & Wilkins.]

Key words: Breast cancer, calmodulin antagonist, estrogen receptor.

Introduction

Endocrine insensitivity is a major clinical challenge in the treatment of breast cancer. Sixty percent of primary breast cancers are estrogen receptor (ER)-positive tumors,[1] of which approximately one-third show an objective response and one-third durable static disease on first-line endocrine therapy.[2] Overall, approximately 40% of tumors exhibit primary resistance to endocrine therapy and are usually ER-negative or, if ER-positive, they exhibit a high rate of cell proliferation.[3] On progression, 10–20% will show an objective response and 30–40% durable static disease to second-line endocrine therapies.[2,4,5] Some endocrine sensitive tumors will go on to respond to third- and even fourth-line endocrine treatments. However, acquired secondary resistance eventually develops in all tumors which show initial sensitivity to endocrine therapies.

The calcium binding protein calmodulin plays a key role in calcium signaling during regulation of cell proliferation[6] and calmodulin antagonists might therefore have some therapeutic potential as anti-proliferative agents.[7–9] Calmodulin antagonists of diverse chemical structures have been reported to inhibit breast cancer cell lines. They include naphthalenesulfonamides,[10–12] calmidazolium[10,12] and pimozide.[11] It has been recognized for some time that the effectiveness of tamoxifen may in part be attributed to its ability to inhibit calmodulin[13,14,15] and rationally designed analogs of tamoxifen with improved calmodulin antagonism have increased cytotoxicity towards MCF-7 cells.[16] The use of calmodulin antagonists, therefore, offers a new approach for treatment of breast cancers which show either primary *de novo* or acquired endocrine resistance. It has recently been reported[17] that a combination of W-7 and tamoxifen induces apoptosis synergistically in breast cancer cells. Calmodulin antagonists could therefore be used in combination with endocrine therapies to assess if this increases therapeutic efficacy against endocrine sensitive cancers.

We now wish to report that a new series of calmodulin antagonists inhibit the growth of breast cancer cells with potency at low micromolar concentrations, comparable with the most active antagonists previously reported.

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The calmodulin antagonists **1**, **2** and **3** are variants of the W series of naphthalenesulfonamides such as W-7[18] and related J compounds, e.g. J-8:[19] their structures are shown in Fig 1. In these new compounds the ω-aminoalkyl side chain is connected to the naphthalene residue by an ether linkage rather than by sulfonamide formation and the terminal nitrogen is part of a strongly basic pyrrolidine ring. The characteristically poor water solubility of the series has been enhanced in compound **3** by the incorporation of a sulfoxide in the side chain. The development of these compounds as potential anti-fungal compounds has been described.[20] Their potency as calmodulin antagonists has been established by a determination of their inhibition of calmodulin-dependent myosin light chain kinase (MLCK) (Table 1).

The inhibitory effect of compounds **1**, **2** and **3** was assessed in parallel with the potent and selective calmodulin antagonist J-8[21] on a panel of human breast cancer cell lines exhibiting differing levels of ER expression (ER-positive MCF-7B, T-47D; ER-low MCF-7T; ER-negative MDA-MB-231). The effect of estradiol (E_2) on the inhibitory effect of the antagonists was also investigated.

Table 1. Calmodulin-dependent MLCK screen

Compound	Mean IC_{50} (μM $\pm$ SEM)	*N*
W-7	48 $\pm$4.9	3
J-8	26 $\pm$2.3	3
1	10.7 $\pm$2.2	6
2	25 $\pm$2.9	3
3	34 $\pm$1.7	3

Materials and methods

Chemicals

Calmodulin antagonists **1**, **2** and **3** were synthesized at Nottingham Trent University,[20] as was *N*-8-aminooctyl-5-iodo-naphthalenesulfonamide, reference compound J-8).[21] *N*-(6-aminohexyl)-5-chloro-1-napthalene sulfonamide hydrochloride (W-7) was obtained from Aldrich (Poole, UK).

Cell lines

The MCF-7 breast cancer cell lines were obtained from Dr J Nelson (Queens University, Belfast) and from the Tenovus Institute (Cardiff). These were designated MCF-7B and MCF-7T, respectively. MCF-7B expresses both estrogen and progesterone receptors (ER and PgR), whereas MCF-7T is a variant which expresses very low levels of ER and PgR.[22] T-47D human breast cancer cell line (which expresses both ER and PgR) and MDA 231 (which is negative for both ER and PgR) were obtained from Professor Carmichael (Clinical Oncology, City Hospital, Nottingham).

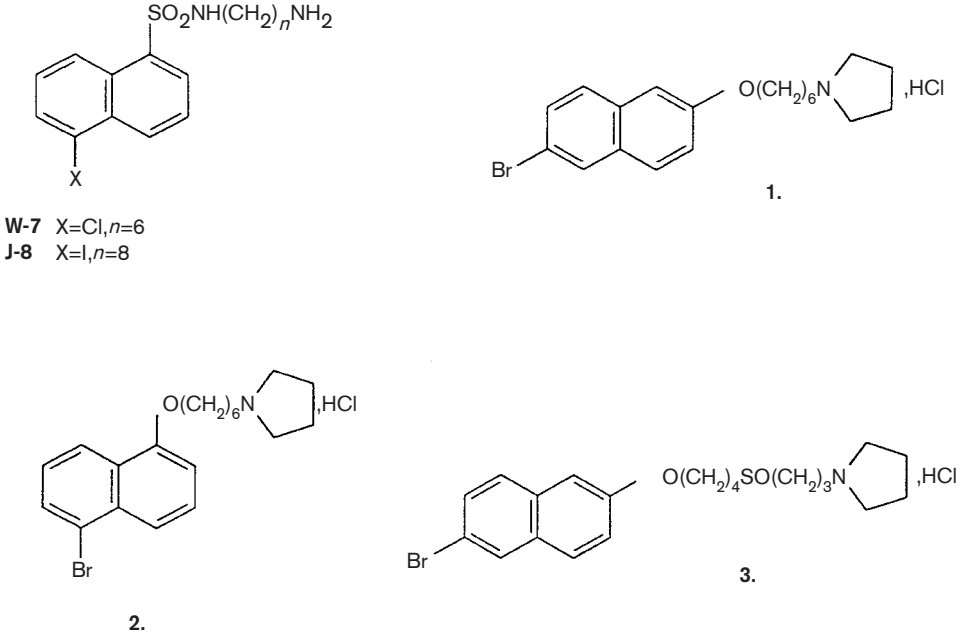

Figure 1. Chemical structures of calmodulin antagonists.

Cell culture and media

Cells were harvested for assay or passage at sub-confluence using 0.025% trypsin–EDTA (Sigma, Poole, UK). Medium for routine cell maintenance was RPMI 1640 with 2 mM glutamine (Sigma) supplemented with 5% heat-inactivated fetal calf serum (FCS; Gibco BRL, Paisley, UK). This normal growth medium contains estrogens (from the serum component) and phenol red which has estrogenic properties. To monitor the effect of E_2, therefore, assays were set up in steroid-depleted medium composed of phenol red-free RPMI 1640 supplemented with 2 mM glutamine plus 5% FCS pre-treated with dextran-coated charcoal to remove endogenous steroids. Cells were grown in this medium for up to 14 days prior to assay to establish basal growth in the absence of estrogen.

Proliferation assays (MTT assay)

Cells were plated at $1–5 \times 10^3$/well (depending on growth characteristics of cell lines) in culture medium in 96-well flat-bottom tissue culture plates. Cells were incubated for 3–4 h to allow cells to adhere, then treatments added. Stock solutions of calmodulin antagonists [1% in dimethylsulfoxide (DMSO) ~2–2.5 mM] were stored at room temperature. Dilutions of these were made immediately before use in assay medium so that the final concentrations of calmodulin antagonists were between 100 μM and 1 nM, and that of DMSO remained constant within each assay (usually 0.2%). E_2 was obtained from Sigma and was used at a final concentration of 1 nM. For each treatment there were three to six replicates in a volume of 200 μl/well. Plates were incubated at 37°C in a 5% CO_2 humidified incubator for 5–14 days until cells in control wells were approaching confluence. MTT (50 μl/well of 1 mg/ml stock) was added for the last 4 h. Medium was aspirated from the wells and replaced with 100 μl DMSO, shaken for 5 min to dissolve formazan crystals, and the absorbance read at 550 nm on an ELISA plate reader. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) is metabolized by viable cells into blue formazan crystals which dissolve in DMSO. The optical density reading (wavelength 550 nm) correlates with the amount of product and therefore the number of viable cells.[23]

Assays were performed at least 3 times in routine culture medium or in steroid depleted medium (see above) with and without E_2 (1 nM). All treatment and control wells within an assay contained the same concentration of DMSO (usually 0.2%). The effect of E_2 on cell proliferation was calculated as a percentage of growth in control wells without E_2 present. The IC_{50} was determined as the concentration of drug where proliferation was 50% of that in relevant control wells. Data from three or four assays for each cell line were pooled for statistical analysis and graphs plotted for IC_{50} determinations. Analysis of variance (ANOVA) was used to determine whether E_2 or calmodulin antagonists affected the proliferation response. Student's *t*-test was applied to determine whether IC_{50} determinations for each CA were lower than those for J-8 for individual cell lines. Values of $p \leqslant 0.05$ were considered significant.

Results

The effect of E_2 on proliferation of breast cancer cell lines

The growth of T-47D and MCF-7B (both ER-positive) cell lines were stimulated by E_2 at nanomolar concentrations ($167 \pm 21\%$, ANOVA, $p=0.014$ and $129 \pm 8\%$, ANOVA, $p=0.006$, respectively) when compared with controls (Figure 2). MCF-7T (ER-low) and MDA-MB-231 (ER-negative) cell lines were not stimulated by E_2 in the same conditions.

Effect of E_2 on determination of IC_{50} for calmodulin antagonists

Five calmodulin antagonists (W-7, J-8, **1**, **2** and **3**) were assayed against four cell lines (MCF-7B, T-47D,

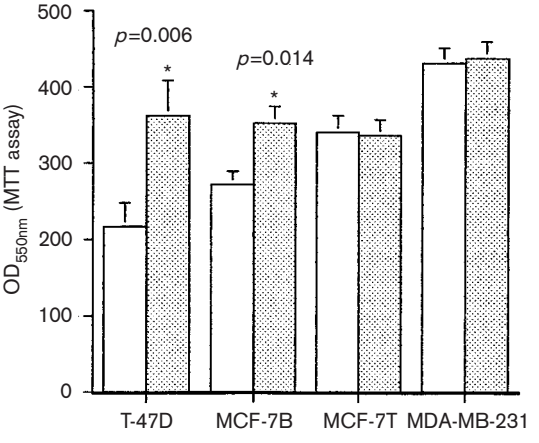

Figure 2. The effect of estrogen on proliferation of breast cancer cell lines. Uptake of MTT by viable cells was measured by reading OD at 550 nm. Mean OD ± SEM in controls (open bars) or in the presence of nM E_2 (shaded bars) are shown for four breast cancer cell lines. Growth of ER-positive cell lines (T-47D and MCF-7B) was significantly stimulated by E_2 (*p values shown). Growth of cells with low ER (MCF-7T) or no ER expression (MDA 231) was not stimulated by E_2.

MCF-7T and MDA-MB-231) in the presence or absence of E_2. Even in the presence of calmodulin antagonists, E_2 continued to stimulate the cell growth of MCF-7B and T-47D, particularly at low calmodulin antagonists concentrations (data not shown). However, the graphs used for determination of IC_{50} with or without E_2 present are virtually superimposable. Representative plots are shown for the determination of IC_{50} of **3** against MCF-7B (Figure 3a) and T-47D (Figure 3b) cell lines. In the case of MCF-7T which has very low ER expression (Figure 3c) and the ER-negative cell line MDA-MB-231 (Figure 3d), E_2 did not stimulate cell proliferation and again both graphs are similar. The findings with respect to response to E_2 and ER status of the breast cancer cell lines were similar for **1** and **2** against the same cell lines. In no case were IC_{50} determinations in the presence of E_2 (proliferation as a percentage of control wells containing E_2) or in the absence of E_2 (proliferation as a percentage of control wells without E_2) significantly different for any calmodulin antagonist against any cell line (Table 2).

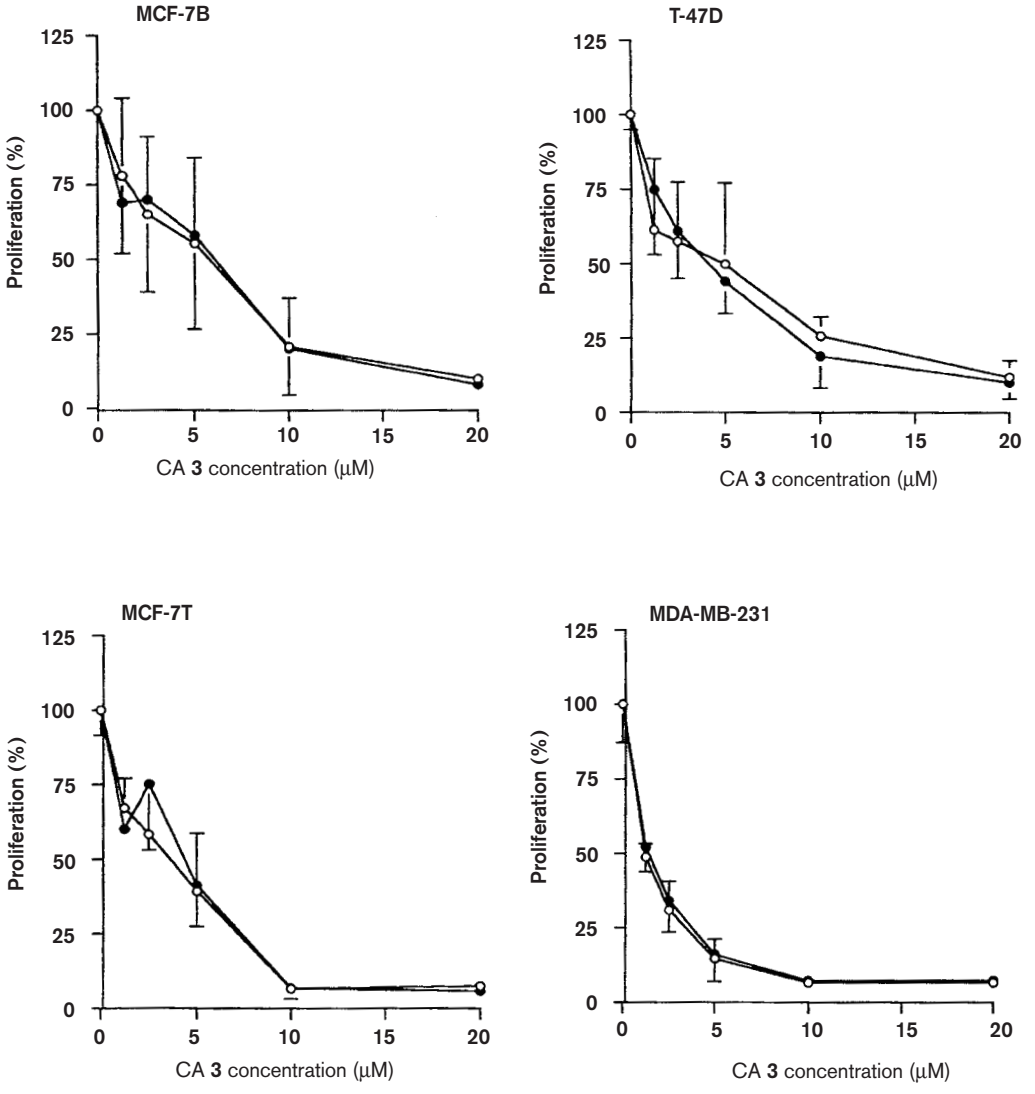

Figure 3. The effect of **3** on proliferation of four human breast cancer cell lines. For IC_{50} determinations without E_2 present, cell proliferation was calculated as a percentage of growth in control wells which did not contain E_2 (open circles, SEM positive error shown). For IC_{50} determinations with E_2 present, cell proliferation was calculated as a percentage of growth in control wells which did contain E_2 (filled circles, SEM negative error shown). Growth of the two ER-positive cell lines, MCF-7B and T-47D, was greater in wells containing E_2 than in equivalent wells without E_2 at low **3** concentrations, but IC_{50} determinations for **3** were similar in the presence or absence of E_2. E_2 had no effect on the growth rate of cell lines expressing low or no ER, MCF-7T and MDA-MB-231, respectively, and IC_{50} determinations for **3** were similar $\pm E_2$.

Table 2. The effect of calmodulin antagonists on proliferation of breast cancer cell lines in the presence or absence of E_2 (nM) [the values shown are mean IC_{50} ($\mu M \pm SEM$) of several assays ($n = 3–6$)]

Cell line		Calmodulin antagonists IC_{50} (μM)				
		W-7	J-8	**1**	**2**	**3**
T-47D	$+E_2$	NT	8.6 ± 1.6	5.7 ± 1.2	4.9 ± 1.3	3.3 ± 0.7
	$-E_2$	NT	8.3 ± 0.9	4.8 ± 2.6 NS	5.3 ± 1.2 $p = 0.021$	4.7 ± 1.8 $p = 0.002$
MCF-7B	$+E_2$	8.3 ± 1.9	7.7 ± 1.3	7.2 ± 1.1	7.7 ± 0.2	5.3 ± 1.2
	$-E_2$	10.0 ± 0.0 NS	9.3 ± 1.1	7.5 ± 1.0 NS	7.3 ± 1.1 NS	5.0 ± 1.6 $p = 0.026$
MCF-7T	$+E_2$	11.6 ± 1.5	9.9 ± 1.4	4.3 ± 0.7	6.6 ± 0.4	4.4 ± 0.7
	$-E_2$	14.0 ± 4.0 NS	11.7 ± 1.5	3.8 ± 0.2 $p = 0.0003$	5.8 ± 0.6 $p = 0.005$	3.8 ± 0.8 $p = 0.0003$
MDA-MB-231	$+E_2$	NT	5.2 ± 1.2	3.0 ± 0.8	3.3 ± 0.7	1.6 ± 0.4
	$-E_2$	NT	8.7 ± 0.7	2.7 ± 1.2 $p = 0.010$	3.2 ± 0.9 $p = 0.014$	1.1 ± 0.1 $p = 0.042$

NT, not tested.
p values are shown where the IC_{50} differs significantly ($p < 0.05$) from that for J-8; $^{NS}p > 0.05$.

The effect of calmodulin antagonists on cell proliferation

Calmodulin antagonists at micromolar concentrations inhibited the growth of four breast cancer cell lines (MCF-7T, MCF-7B, MDA-MB-231 and T-47D). IC_{50} values (mean $\pm$ SEM of determinations with or without E_2) for five calmodulin antagonists are shown in Table 2

The reference compound W-7 was tested against MCF-7B and MCF-7T cell lines (expressing high or low levels of ER, respectively), and was of similar efficacy to J-8 ($p > 0.05$), with IC_{50} values in the 8–14 μM range. IC_{50} values obtained for the three new calmodulin antagonists were consistently lower than those for J-8. The difference was significant ($p < 0.05$) for **1** against two cell lines (MCF-7T and MDA-MB-231) and **2** against three of the four cell lines (MCF-7T, T-47D and MDA-MB-231). Calmodulin antagonist **3** performed best, being approximately twice as potent as J-8 with mean IC_{50} values of 3–5 μM or less against all four breast cancer cell lines tested.

Discussion

There is a good correlation between initial endocrine sensitivity and levels of ER expression in human breast cancer.[1,3] Two variants of the cell line (MCF-7) used in these assays express different ER phenotypes.[22] This is reflected in their differing endocrine sensitivity evidenced by their proliferation response to E_2 in *in vitro* culture. MCF-7B and T-47D both express ER and were stimulated by physiological levels of E_2, whereas MCF-7T, which has low ER expression, and ER-negative breast cancer cell lines, such as MDA-MB-231, were not.

The efficacy of calmodulin antagonists W-7, J-8 and their derivatives was comparable against human breast cancer cell lines exhibiting both ER-positive and -negative phenotypes, and was not altered in conditions where E_2 stimulated proliferation of E_2-sensitive cell lines. Strobl *et al.*[24] have reviewed the possible role of calmodulin antagonists in the inhibition of breast cancer cell growth. They cite evidence that the rank order of potency in the inhibition of MCF-7 cell growth parallels that of the sensitivity of calmodulin towards the inhibitors and that the antiproliferative activity of calmodulin antagonists appears to be independent of the estrogen response pathways.

The results of this study are in accord with these conclusions. These calmodulin antagonists, at micromolar concentrations, effectively inhibited growth of breast cancer cells regardless of their ER status. The new antagonists **1**, **2** and **3** and the sulfonamide J-8 are all more potent inhibitors of calmodulin-dependent MLCK than is W-7 (Table 1) and all also show greater antiproliferative activity.

Compound J-8 is a good inhibitor of calmodulin-dependent phosphodiesterase (IC_{50} of 3 μm), which has been shown to have anti-proliferative properties against K562 human leukemic lymphocyte cells[21] and

to inhibit invasion of a human melanoma cell line through fibronectin.[25] The antagonists **1** and **2** are significantly more active than J-8 against the whole range of breast cancer cell lines, while the sulfoxide-containing **3**, less active in the MLCK screen, proved to be equal with **1** in the whole cell assays, perhaps because of its increased solubility in aqueous media. It is noteworthy that **1** and **2**, which are highly hydrophobic, exhibited micromolecular inhibitory activity in a medium containing calf serum, where protein binding of the compounds by the medium must significantly lower their effective concentration.

Conclusion

Calmodulin antagonists represent an alternative therapeutic approach for the treatment of breast cancer. They could potentially play an important role both in the treatment of primary hormone resistance and also in the treatment of acquired resistance. Further investigation is merited on both these fronts. Novel calmodulin antagonists, as described here, may in the future offer an additional therapeutic strategy for the treatment of breast cancer.

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