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# A SURVEY OF HUMAN BREAST CANCER SENSITIVITY TO GROWTH INHIBITION BY CALMODULIN ANTAGONISTS IN TISSUE CULTURE

JEANNINE S. STROBL,* VIRGINIA A. PETERSON and KAREN A. WOODFORK

Department of Pharmacology and Toxicology, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26506-9223, U.S.A.

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**Abstract**—We compared the ability of *N*-(4-aminobutyl)-5-chloro-2-napthalenesulfonamide (W-13), a calmodulin antagonist, to inhibit the growth of seven human breast cancer cell lines in tissue culture, to determine whether drug sensitivity was related to estrogen receptor (ER) status, tamoxifen resistance (tam$^r$), or levels of calmodulin activity. We examined three ER$^+$ (estrogen receptor-positive) cell lines (MCF-7, ZR-75-1B, and T47D), two ER$^+$/tam$^r$ lines (LY2 and RR), and two ER$^-$ (estrogen receptor-negative) cell lines (MDA-MB-231 and MDA-MB-435). There was no difference in the inhibition of cell growth by W-13 in MCF-7 cells and the two tam$^r$ MCF-7 cell derivatives, LY2 and RR. In addition, the sensitivity to W-13 did not appear to be related to ER status. Although the mean $K_i$ of the five ER$^+$ cell lines (31 $\mu$M) was somewhat higher than the mean $K_i$ of the two ER$^-$ cell lines (23 $\mu$M), the two cell lines most sensitive to W-13 were the ER$^+$ T47D cells ($K_i$ 15 $\mu$M) and the ER$^-$ MDA-MB-435 cells ($K_i$ 10 $\mu$M). Calmodulin activity was measured in three representative cell lines, MCF-7, LY2, and MDA-MB-435. Calmodulin levels were higher in the most sensitive cell line (MDA-MB-435, 2.7 ng calmodulin/$\mu$g protein) than in the two less sensitive cell lines, MCF-7 and LY2 (1.3 and 1.6 ng calmodulin/$\mu$g protein, respectively). However, the MCF-7, LY2, and MDA-MB-435 cells were equally sensitive to another specific calmodulin antagonist, calmidazolium. We conclude that neither ER status, tamoxifen resistance, nor levels of calmodulin activity predict the sensitivity of human breast cancer cell lines to growth inhibition in tissue culture by calmodulin antagonists.

*Key words:* breast cancer; calmidazolium; calmodulin; MCF-7; tissue culture; W-13

Calmodulin is a ubiquitous calcium-binding protein that mediates many intracellular calcium-dependent processes [1]. Calmodulin activation plays an essential role in DNA synthesis and mitosis [2, 3], and pharmacological inhibition of calmodulin activation has been investigated as a means for controlling the proliferation of cancer cells [4]. Human mammary tumor cells in tissue culture are highly sensitive to the growth inhibitory actions of calmodulin. The calmodulin selective agents, W-7, W-13† and calmidazolium (R24571), phenothiazine calmodulin antagonists such as trifluoperazine, fluphenazine and thioridazine, and the diphenylbutylpiperidine neuroleptic agent, pimozide, all exert antiproliferative effects on both ER$^+$ and ER$^-$ human breast cancer cell lines at concentrations consistent with an action on calmodulin [5–7]. Biochemical assays have demonstrated that calmodulin activation of the calcium-dependent phosphodiesterase activity is inhibited by a large number of neuroleptic agents [8]. Interestingly, the incidence of cancer, including breast cancer, is reduced among patients chronically treated with neuroleptic drugs [9, 10]. These data suggest that calmodulin antagonism is a potentially useful therapeutic mode of anticancer drug action. However, reports of the clinical application of calmodulin antagonists in cancer chemotherapy are limited [11, 12]. The present study sought to identify breast tumor types that might exhibit special sensitivity to growth inhibition by calmodulin antagonists.

* Corresponding author: Dr. Jeannine Strobl, Department of Pharmacology and Toxicology, West Virginia University, P.O. Box 9223, Morgantown, WV 26506-9223. Tel. (304) 293-7151; FAX (304) 293-6854.

† Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; ER, estrogen receptor; MOPS, 3-[*N*-morpholino] propanesulfonic acid; tam$^r$, tamoxifen-resistant; and W-13, *N*-(4-aminobutyl)-5-chloro-2-napthalenesulfonamide.

## MATERIALS AND METHODS

*Drugs.* W-13, purchased from Seikagaku America, Inc. (Rockville, MD), was dissolved in sterile water at a 1000× concentration and stored at 4°. Calmidazolium (Janssen Pharmaceutica Inc., Piscataway, NJ) was dissolved in sterile water and used immediately.

*Cell culture.* The ER-positive cell lines used were MCF-7 (passage 36–60) [13], ZR-75-1B [14], and T47D [15]. Two tamoxifen-resistant derivatives of the MCF-7 cell line were studied, LY2 [16] and RR [17]. The RR line is also retinoic acid resistant [18]. The ER-negative cells used in these experiments were the MDA-MB-231 [19] and MDA-MB-435 [20] lines. All cell lines were grown in DMEM (Bio-Whittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY), 2 mM glutamine and 40 $\mu$g/mL

gentamicin. Cells were maintained at 37° in a humidified 5% $CO_2$/95% $O_2$ atmosphere. MCF-7 and ZR-75-1B cells were passaged weekly at a 1:5 ratio. All other cell lines were passaged weekly at a 1:10 ratio.

*Growth inhibition assays.* Confluent monolayers of cells were harvested by trypsinization and plated in triplicate at a density of $2.8 \times 10^5$ cells/35 mm$^2$ dish in DMEM containing 10% fetal bovine serum, glutamine, gentamicin and the specified concentration of W-13 or calmidazolium. Sterile water was added to control cells. Cells were incubated at 37° for 48 hr; then cells in the medium and attached to the dish were harvested and counted. Cell viability was assessed by trypan blue (5%) exclusion. The inhibition constants ($K_i$) were derived from the cell count data using the equation: $K_i = (1/f_b - 1)$ (drug concn), where $f_b$ = the fractional drug response.

*Calmodulin assays.* Calmodulin activity was measured in extracts of the breast cancer cell lines using the $Ca^{2+}$-dependent phosphodiesterase activity assay [21]. Cells were plated and incubated for 48 hr in the absence of drug, as described above for the growth inhibition assays. After harvesting, the cells were rinsed once with Hanks' Balanced Salt Solution, and collected by centrifugation (1000 *g*, 5 min). The cell pellets were resuspended ($4.6 \times 10^7$ cells/2 mL) in 125 mM borate buffer, 5 mM EGTA, 75 mM NaCl, pH 8.4, placed on ice, and disrupted by sonication. These homogenates were heated at 90° for 3 min to release membrane bound calmodulin, and then quick-chilled on ice. Cell extracts were prepared by centrifugation (10,000 *g*, 30 min, 4°). Aliquots were removed for total protein determinations [22]; then the extracts were stored frozen at −20° overnight.

Enzyme reactions contained 50 mM MOPS, pH 7.0, 12 mM magnesium acetate, 20 mM 2-mercaptoethanol, 0.4 mM $CaCl_2$, 0.1 μg/mL bovine serum albumin, and 20 μg 3′, 5′-cyclic nucleotide phosphodiesterase (activator-deficient from bovine heart) (3′,5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) (Sigma, St. Louis, MO). A heat-inactivated calmodulin standard, 0.2 to 10 ng, phosphodiesterase 3′, 5′-cyclic nucleotide activator purified from bovine brain (Sigma), or cell extract (diluted 1:20 to 1:30 with the sonication buffer) was added to the reaction mix, and was preincubated for 10 min at 30°. Reactions were initiated by the addition of [$^3$H]adenosine 3′, 5′-cyclic phosphate to a final concentration of 30 μM (sp. act. 2.5 Ci/mmol, New England Nuclear, Boston, MA), and run for 15 min at 30°. Reaction kinetics were linear under these conditions. After termination of the phosphodiesterase reaction, a 15-min incubation with 30 μg 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) from *Crotalus atrox* (Sigma) converted the reaction product to [$^3$H]-adenosine. [$^3$H]Adenosine was identified and quantified by liquid scintillation counting following separation of the reaction products by thin-layer chromatography on PEI-cellulose plates (Eastman Kodak Co., Rochester, NY) developed in 1 M ammonium acetate:95% ethanol (3:7, v/v).

*Statistical analysis.* Data were analyzed by a one-way analysis of variance and Newman–Keul's *t*-test for multiple pairwise comparisons. The level of statistical significance was set at $P < 0.05$.

Table 1. W-13 inhibition of cell growth

| Cell line | Cell phenotype* | $K_i$ (μM) | N |
|---|---|---|---|
| MCF-7 | ER$^+$ | 39.2 ± 7.8 | 5 |
| ZR-75-1B | ER$^+$ | 22.0 ± 5.8† | 4 |
| T47D | ER$^+$ | 14.7 ± 4.4† | 4 |
| LY2 | ER$^+$/tam$^r$ | 42.2 ± 12.0 | 3 |
| RR | ER$^+$/tam$^r$ | 35.3 ± 16.2 | 3 |
| MDA-MB-231 | ER$^-$ | 35.4 ± 13.2 | 4 |
| MDA-MB-435 | ER$^-$ | 10.2 ± 4.3† | 3 |

Cells were plated in DMEM plus 10% fetal bovine serum at a density of $2.1 \times 10^4$ cells/cm$^2$ and grown for 48 hr in the presence of 0–94 μM W-13. Viable cells were counted, and $K_i$ values were calculated as described in Materials and Methods. Data are the means ± SEM of N independent experiments performed in triplicate.

* ER$^+$, estrogen receptor-positive; ER$^-$, estrogen receptor-negative; and tam$^r$, tamoxifen-resistant.

† Significantly different from other cell lines, $P < 0.05$.

## RESULTS

*Growth inhibition of ER$^+$ and ER$^-$ cell lines by W-13.* The growth of three ER$^+$ and two ER$^-$ human breast cancer cell lines was inhibited in a concentration-dependent fashion by W-13. The inhibition constants calculated from these results are summarized in Table 1, and indicate that the maximum difference in sensitivity to W-13 among these five cell lines was 4-fold. The ER$^+$ MCF-7 cell line was the least sensitive to W-13 and the ER$^-$ MDA-MB-435 cell line was the most sensitive. However, the similar sensitivities of the MCF-7, ZR-75-1B, and MDA-MB-231 cells to W-13 indicate that growth inhibition by this calmodulin antagonist is not predictable on the basis of ER status.

The decreases in cell number with increasing W-13 concentration were closely paralleled by reductions in the percentage of viable cells (data not shown), indicating that the action of W-13 in these cells is cytotoxic, not cytostatic.

*Growth inhibition of tam$^r$ cell lines by W-13.* The LY2 and RR cell lines are tamoxifen-resistant derivatives of the MCF-7 cell line. Since the mechanism of tamoxifen resistance in these lines is not clearly understood, it was of interest to determine whether tamoxifen resistance was accompanied by resistance to calmodulin antagonism. The LY2 and RR cell lines retained sensitivity to growth inhibition by W-13. The inhibition constants for W-13 were indistinguishable among these three cell lines (Table 1). W-13 was cytotoxic in these cell lines, as determined by cell viability assays (data not shown).

The LY2 cell line exhibited a consistent growth stimulation at low concentrations of W-13, which was not observed in the MCF-7 or RR cell lines. The basis of this growth stimulation was not clear.

Table 2. Calmidazolium inhibition of cell growth

| Cell line | $K_i$ ($\mu$M) |
|---|---|
| MCF-7 | 3.8 ± 0.88 |
| LY2 | 4.5 ± 0.50 |
| MDA-MB-435 | 3.4 ± 1.4 |

Cells were plated in DMEM plus 10% fetal bovine serum at a density of $2.1 \times 10^4$ cells/cm$^2$ and grown for 48 hr in the presence of 0–10 $\mu$M calmidazolium. Viable cells were counted, and $K_i$ values were calculated as described in Materials and Methods. Data are the means ± SEM of 4 independent experiments performed in triplicate.

*Growth inhibition by calmidazolium.* A second selective calmodulin antagonist, calmidazolium, was used to test further the idea that ER status and tamoxifen resistance cannot be used to predict the sensitivity of human breast cancer cells to calmodulin antagonists. Three cell lines were chosen for these studies, the ER$^+$ MCF-7 cells and the ER$^-$ MDA-MB-435 cells, representing the extremes in sensitivity to W-13, and the tamoxifen-resistant LY2 line. The growth of all three cell lines was inhibited to a similar extent by calmidazolium (Table 2). As with W-13, reductions in cell number with increasing drug concentration caused proportional decreases in cell viability. Thus, calmodulin antagonists caused cell death in all of these human breast cancer cell lines at the concentrations used in these studies.

Calmidazolium was a more potent inhibitor of growth than W-13. MCF-7 and LY2 cells exhibited a 10-fold lower $K_i$ value for calmidazolium than for W-13. Similarly, the MDA-MB-435 cells were highly sensitive to growth inhibition by calmidazolium, and the $K_i$ was approximately 3-fold lower for calmidazolium than for W-13.

*Calmodulin levels in human breast cancer cell lines.* Levels of biologically active calmodulin were compared in MCF-7 and MDA-MB-435 cells to determine whether differences in calmodulin levels were apparent in ER$^+$ versus ER$^-$ human breast cancer cell lines. The results, summarized in Table 3, show that the ER$^-$ MDA-MB-435 cell line exhibited a 2-fold greater level of biologically active calmodulin than the ER$^+$ MCF-7 human breast cancer cells. This result does not agree with an earlier study showing that ER$^+$ tumors had more calmodulin than ER$^-$ human breast tumors [23]. However, in this previous study, total calmodulin, not biologically active calmodulin, was measured.

Table 3. Calmodulin activity

| Cell line | Calmodulin (ng/$\mu$g protein) | N |
|---|---|---|
| MCF-7 | 1.3 ± 0.22 | 5 |
| LY2 | 1.6 ± 0.22 | 5 |
| MDA-MB-435 | 2.7 ± 0.28* | 3 |

Cells were plated in DMEM plus 10% fetal bovine serum at a density of $2.1 \times 10^4$ cells/cm$^2$ and grown for 48 hr in the absence of drugs. Cells were harvested, and supernatants were prepared for calmodulin assay. Biologically active calmodulin was measured using the activation of $Ca^{2+}$-dependent phosphodiesterase. Values are the means ± SEM of N independent experiments performed in duplicate.

* Significantly different ($P < 0.05$) from both the MCF-7 and LY2 cells.

Tamoxifen can inhibit calmodulin activation, and perhaps this contributes to the inhibition of cell growth [24]. Levels of calmodulin in the LY2 cells were examined to test whether tamoxifen resistance was accompanied by an increase in calmodulin activity. The data in Table 3 show that biologically active calmodulin was not increased significantly in the LY2 cells as compared with the parental MCF-7 cell line.

## DISCUSSION

The development of calmodulin antagonists for use in cancer therapy is of interest because calmodulin represents an essential, non-DNA target for inhibition of proliferation [1–4]. We previously published evidence indicating that ER$^-$ human breast cancer cell lines may be more sensitive than ER$^+$ human breast cancer cell lines to growth inhibition by calmodulin antagonists [6]. A 72 hr incubation with 35 $\mu$M W-13 caused a 100% inhibition of MDA-MB-435 cell numbers, but only a 40–50% decrease in the number of MCF-7 cells. In a study of human breast cancer tumor samples, Krishnaraju *et al.* [23] determined that calmodulin levels were positively correlated with the presence of ER. Among 38 tumor samples, levels of total calmodulin protein in the ER$^+$ tumors were approximately twice those in ER$^-$ tumors. On the basis of these observations, we hypothesized that slight elevations in calmodulin levels in ER$^+$ breast cancers could provide a survival advantage during growth in the presence of calmodulin antagonists. We demonstrated that although the ER$^-$ MDA-MB-435 human breast cancer cell line was 4-fold more sensitive to growth inhibition by W-13 than ER$^+$ MCF-7 cells, other ER$^+$ human breast cancer cell lines were as sensitive to W-13 as the ER$^-$ lines tested. Furthermore, MCF-7 and MDA-MB-435 cells were equally sensitive to growth inhibition by another calmodulin antagonist, calmidazolium.

An enzymatic assay for calmodulin, detecting only biologically active calmodulin, was used to measure calmodulin levels in the human breast cancer cells under the same conditions that the growth studies were performed. Levels of calmodulin found in the human breast cancer cell lines were within the range expected for normal (non-brain) tissues [21]. All three cell lines contained endogenous inhibitors of calmodulin activity, and maximal activity was observed only upon dilution of the cell extract. After diluting to obtain maximal activity, the ER$^-$ MDA-MB-435 cell line exhibited the most calmodulin activity, twice that of the MCF-7 cell line, and LY2 cells exhibited an intermediate level of calmodulin. Thus, the MDA-MB-435 cells, which exhibited the

greatest sensitivity to both W-13 and calmidazolium, expressed the highest levels of calmodulin activity. Perhaps it is because these cells require higher calmodulin levels for growth that they are the most sensitive to calmodulin antagonists. It was not possible to predict sensitivity to calmodulin antagonists by measurements of calmodulin alone, however, since MCF-7 and LY2 cells exhibited statistically significantly less calmodulin activity but were as sensitive as MDA-MB-435 cells to calmidazolium.

Tamoxifen is an antiestrogen that is active primarily in the treatment of $ER^+$ human tumors [25], but actions unrelated to the estrogen receptor have been observed, and these may contribute to the therapeutic effectiveness of tamoxifen. Antiestrogenic concentrations of tamoxifen ($\geq 1\ \mu M$) inhibit calmodulin-dependent phosphodiesterase activity [24]. Tamoxifen, as well as other calmodulin antagonists, can reverse the multidrug-resistant phenotype [26, 27]. Tamoxifen has also been shown to interfere with the association of estrogen receptors with calmodulin [28]. The mechanism of the tamoxifen-resistant phenotype of the LY2 cells is unknown, since the ER in these cells is apparently normal [29]. No differences in the levels of calmodulin activity were seen in MCF-7 and LY2 cells, suggesting that alterations in calmodulin activity, at least as measurable in cell extracts, cannot account for the tamoxifen resistance.

In conclusion, to increase the potential effectiveness of calmodulin antagonists in the treatment of human breast cancer, we attempted to identify convenient assays to predict sensitivity to calmodulin antagonists, e.g. ER status, tamoxifen sensitivity, and calmodulin activity. We found that none of these factors alone predicted sensitivity to growth inhibition by W-13 and calmidazolium in human breast cancer cells in tissue culture.

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