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Inhibition of growth of leukemic cells by inhibitors of calmodulin: Phenothiazines and melittin

William N. Hait[1], Linda Grais, Constance Benz[2], and Ed. C. Cadman[2]

[1] Yale University School of Medicine, Department of Medical Oncology, New Haven, CT 06510, USA, and
[2] Cancer Research Institute, University of California at San Francisco, San Francisco, CA, USA

Summary. *Calmodulin, a ubiquitous calcium-binding protein, has recently been shown to play an important role in cellular proliferation. The calmodulin inhibitors melittin, trifluoperazine, and chlorpromazine inhibited the growth and clonogenicity of human and murine leukemic cells, and their potency reflected their activity as inhibitors of calmodulin. Melittin, which is a far more potent inhibitor of calmodulin activity, was also a more potent inhibitor of cell growth and clonogenicity. The less active phenothiazine metabolite, chlorpromazine sulfoxide, had much less potent cytotoxic activity.*

Introduction

Calmodulin is a heat-stable, calcium-binding protein that regulates a wide variety of calcium-dependent processes [7, 32]. Recent evidence suggests that calcium and calmodulin play a crucial role in cellular proliferation [14, 22]. For example, calmodulin changes in concentration during the cell cycle, being greatest between the $G_{1/S}$ phases [5]; stimulates DNA synthesis in rat hepatocytes [2]; and appears to participate in the construction of the mitotic apparatus [1, 8, 33]. An alteration of calmodulin activity has been associated with abnormal cellular proliferation. Accordingly, the concentration of calmodulin is increased in transformed cells [4, 29] and in rapidly growing Morris hepatomas [21, 30].

Hait and Weiss have shown that calmodulin is present in high concentrations in L1210 and L5178Y leukemic cells, and that its activity could be inhibited by phenothiazines [12]. Calmodulin has now been isolated from a variety of malignant cells and found to have properties similar to that of the protein isolated from cerebrum [12, 18]. Recently, MacManus et al. have identified a thus far tumor-specific calcium-binding protein, termed oncomodulin [19]. Although similar to calmodulin in its ability to activate cyclic nucleotide phosphodiesterase and in its inhibition by phenothiazines, it is smaller (Mr 11 500) and more acidic (Pi3.9), and has fewer calcium-binding sites (2 per molecule) [9, 20].

Drugs that inhibit the activity of calmodulin have been shown to inhibit DNA synthesis in a glioblastoma cell line [23], block the movement of chromosomes during metaphase [22], inhibit the growth of Chinese hamster ovary cells [5], and enhance the cytotoxicity of vincristine and doxorubicin [10, 27]. Recent evidence suggests that calmodulin inhibitors are cytotoxic to malignant cells both in vitro [13, 31] and in vivo [15].

Offprint requests to: W. N. Hait

Following the seminal observation by Levin and Weiss [17] that phenothiazines and structurally related compounds inhibit the activity of calmodulin, more potent inhibitors have been identified [25, 26]. Specifically, melittin, a major polypeptide component of bee venom was one of the most potent of these agents [26]. We now report that several drugs that inhibit the activity of calmodulin inhibit the growth of both murine and human leukemic cells and that melittin has the most potent cytotoxic activity.

Materials and methods

L1210 and L5178Y murine leukemic lymphocytes and HL-60 human promyelocytic leukemic granulocytes were grown in suspension culture at 37° C in a 5% CO_2 atmosphere. Tests for contamination with mycoplasma were done monthly. For all experiments cells were inoculated at a density of 1×10^4 cells/ml in Fischer's medium or RPMI-1640 supplemented with 10% horse serum containing a wide range of concentration of drugs. All experiments were designed to compare the various inhibitors of calmodulin on the same days, under identical conditions, during the logarithmic phase of growth. Cells were counted daily using an automated electronic counter (Coulter).

The effect of inhibitors of calmodulin on the clonogenicity of cells was evaluated. After exposure to drug for 48–72 h, cells were washed in fresh medium by centrifugation at 100 *g* for 10 min, then cloned in drug-free medium containing soft agar as previously described [3]. The colonies formed were counted 14 days later using a dissecting microscope with a scored field. Efficiency of cloning ranged from 70% to 80%.

Calmodulin was prepared from L1210 cells by a modification of previously described methods [12, 28]. In this procedure, 1×10^9 L1210 cells were suspended in a phosphate-buffered solution containing 88 m*M* NaH_2PO_4, 23 m*M* Na_2HPO_4, 640 m*M* glycine, 1 m*M* ethylene glycol-bis (B-aminoethylether)-*N,N'*-tetraacetic acid (EGTA), and 0.32 *M* sucrose, pH 6.0. The cells were sonicated at 50 W for 10 s, incubated at 37° C for 2 h, placed in a boiling water bath for 5 min, and then centrifuged at 3,600 *g* for 20 min. The supernatant fraction was removed, the pH was adjusted to 6.0, and the cells were recentrifuged as above. The supernatant fraction was dialyzed for 12 h against the sonicating buffer then concentrated to one-half its original volume by ultrafiltration through a CX-10 filter (Millipore Corp., Bedford, Mass).

A 7.5% preparative polyacrylamide gel electrophoretic column (Shandon Southern Instruments, Inc., Sewickley, Pa)

was prepared in a phosphate buffer solution (pH 7.0) containing 39 m*M* NaH_2PO_4, 123 m*M* Na_2HPO_4, 640 m*M* glycine, 0.46 ml TEMED, and 1 m*M* EGTA. An electrolyte buffer was prepared using 88 m*M* NaH_2PO_4, 23 m*M* Na_2HPO_4, 640 m*M* glycine, and 1 m*M* EGTA. After this, 2 ml of partially purified calmodulin was applied to the gel column and the electrophoresis begun at 25 mA for the 1st hour and 60 mA thereafter, using a constant current power supply (BIO-RAD Laboratories, Model 500/200, Richmond, Calif). Proteins were eluted with a solution of 0.1 *M* Tris buffer (pH 7.6) containing 1.0 m*M* $CaCl_2$ at a flow rate of 0.2 ml/min. One hundred 1-ml fractions were collected and each fraction was assayed for calmodulin activity. Using this method, a single peak of calmodulin was obtained eluting in fractions 35–50 and was found to be homogeneous by SDS-polyacrylamide gel electrophoresis.

The activity of calmodulin was assayed by its ability to activate a calmodulin-sensitive phosphodiesterase prepared from rat cerebrum as previously described [12]. Using this preparation the activity of phosphodiesterase was increased 5–10 fold. One unit of activity was defined as the amount of calmodulin required to achieve 50% of the maximum activation of phosphodiesterase. The effect of inhibitors and their solvents on calmodulin-dependent activity of the enzyme was measured by the ability to inhibit the activation of phosphodiesterase in the presence of 10 U of calmodulin.

Melittin was obtained from the Sigma Chemical Company, St. Louis, Missouri. Chlorpromazine, chlorpromazine sulfoxide, and trifluoperazine were gifts from Dr Alfred Brown of the Smith Kline and French Laboratories, Philadelphia. Drugs were dissolved in distilled water and sterilized by passage through a millipore filter.

Results and discussion

Figure 1 compares the effect of three calmodulin inhibitors on the proliferation of L1210 leukemic lymphocytes. Table 1 demonstrates that the order of potency of inhibition of cell growth corresponded to the order of potency of inhibition of calmodulin purified from this cell line. Accordingly, melittin was a more potent inhibitor of both the activity of calmodulin and the growth of L1210 cells compared with that of trifluoperazine and chlorpromazine.

Chlorpromazine sulfoxide is a metabolite of the parent molecule having far less activity as an inhibitor of calmodulin from cerebrum [17] and L1210 cells (Table 1). Figure 2 demonstrates that the parent compound was approximately 15-fold more potent as an inhibitor of the growth of L1210 cells than the metabolite.

The differences in the activity of melittin compared to the phenothiazines could not be attributed to differences in the shape of the growth curves, since we compared the effect of these drugs daily over a 72-h period and found the observed differences in drug potency on each day of determination (Table 2).

Since the number of cells present in culture is a reflection of both proliferation and death of cells, the inhibition of growth by calmodulin inhibitors could be due to inhibition of proliferation rather than to cytotoxicity. Therefore, we grew cells in the presence of the inhibitor for 48 h, then washed the cells by gentle centrifugation to remove remaining drug and assayed their ability to form colonies in soft agar. All drugs were found to be potent inhibitors of clonogenicity. Melittin

Table 1. Effect of drugs on the inhibition of the activity of calmodulin and the growth of L1210 leukemic lymphocytes

	Inhibition of calmodulin (IC_{50}, μM)[a]	Inhibition of cell growth (IC_{50}, μM)
Melittin	0.12	<1
Trifluoperazine	10	5
Chlorpromazine	15	6
Chlorpromazine-sulfoxide	>300	100

[a] IC_{50} for the inhibition of calmodulin is the concentration of drug needed to produce 50% inhibition of the activation of phosphodiesterase in the presence of 10 U of calmodulin and was calculated from dose-response curves having six determinations at each point. The IC_{50} value for inhibition of cell growth is the concentration of drug needed to produce 50% inhibition of cell growth compared to untreated controls as determined from dose-response curves from three separate experiments as described in *Methods* and Fig. 1

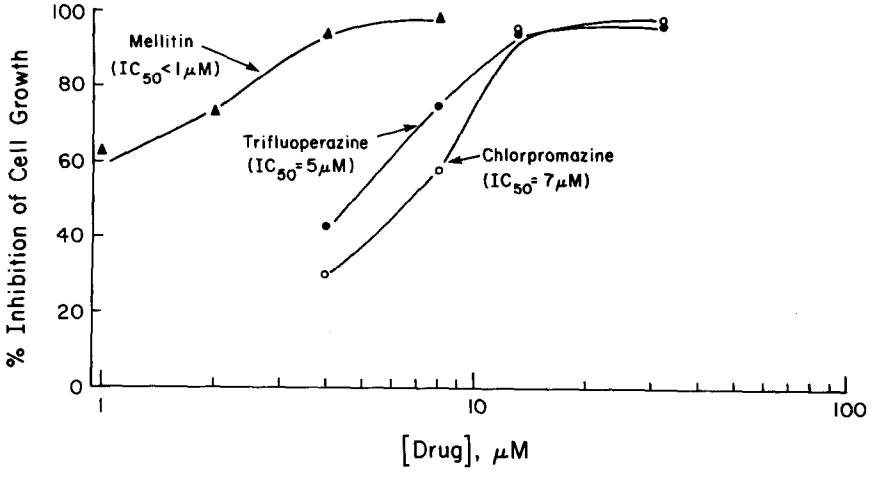

Fig. 1. Effect of calmodulin inhibitors on the growth of L1210 leukemic lymphocytes. Cells were grown in Fischer's medium supplemented with 10% horse serum and maintained in a humidified incubator having a 5% CO_2 atmosphere at 37° C. Cells were inoculated at a density of 1×10^4 cells/ml into 5-ml plastic culture tubes with media containing different concentrations of drugs. Cells were counted at 72 h of growth using a Coulter counter (Hialeah, Fla). Each *point* represents the mean number of cells from three culture tubes from a representative of three experiments. The control cultures contained 3.0×10^5 cells/ml. There was less than 10% variation in cell counts at each point

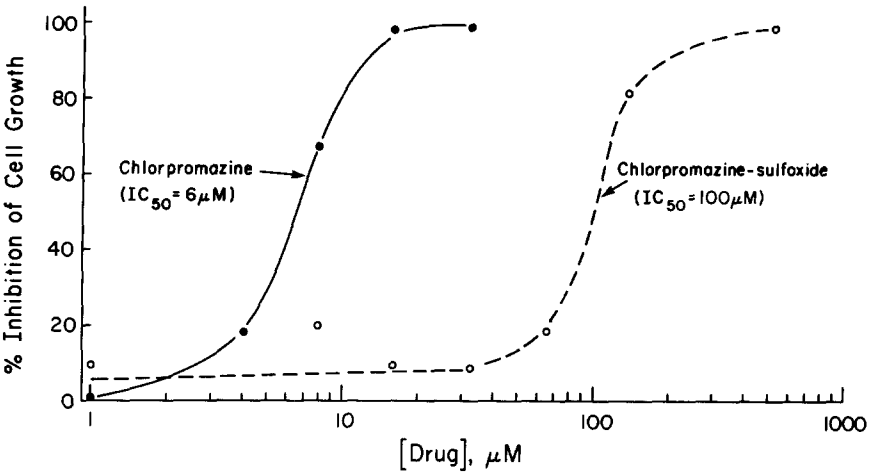

Fig. 2. Effect of chlorpromazine and chlorpromazine sulfoxide on the growth of L1210 leukemic lymphocytes. Cells were grown and drugs prepared as described in Figure 1. Each *point* represents the mean number of cells from three culture tubes from a representative of three experiments. There was less than 10% variation in cell counts at each point. The control cultures contained 4.0×10^5 cells/ml

Table 2. The effect of phenothiazine and polypeptide calmodulin inhibitors on the daily growth of L1210 leukemic lymphocytes

Days of growth	Inhibition of growth (IC_{50}, μM)[a]	
	Trifluoperazine	Melittin
1	> 20	2.5 ± 0.2
2	8.7 ± 0.6	0.7 ± 0.1
3	7.3 ± 0.1	0.8 ± 0.1

[a] IC_{50} value is the concentration of drug needed to inhibit the growth of cells by 50% compared with that of vehicle-treated controls. Each value represents the mean ± SEM of three separate dose-response curves using drug concentrations ranging from 1 to 20 μM

Table 3. Effect of chlorpromazine and chlorpromazine sulfoxide on the clonogenicity of human and murine leukemic cells[a]

Cell line	Drug	
	Chlorpromazine (IC_{50}, μM)	Chlorpromazine sulfoxide (IC_{50}, μM)
L1210	6	90
L5178Y	7	90
HL-60	8	100

[a] Cloning efficiency was 80%. The concentration of drug required to produce 50% inhibition of growth of colonies (IC_{50}) was calculated from dose response curves using the mean of six determinations for each concentration of drug. There was less than 10% variation between culture tubes

was a more potent inhibitor of the clonogenicity of L1210 cells (IC_{50} = 0.7 μM) than trifluoperazine (IC_{50} = 4 μM).

Since calmodulin is present in a variety of malignant cells, we tested whether these drugs would also inhibit the growth of other leukemic cell lines (Table 3). These studies demonstrate that chlorpromazine also inhibited the growth of L5178Y leukemic lymphocytes and the human promyelocytic cell line, HL-60. Again, chlorpromazine sulfoxide had far less inhibitory activity.

Although calmodulin inhibitors are useful tools for elucidating the actions of this protein, they have other pharmacological actions, and therefore the results of these and other studies must be interpreted with caution. However, there is evidence to support the concept that these drugs exert their effects on cellular proliferation through the inhibition of calmodulin. For example: (1) Structurally unrelated substances sharing the property of calmodulin inhibition were also cytotoxic. Thus, in addition to the polypeptide, melittin, and the phenothiazines, trifluoperazine, and chlorpromazine, other classes of calmodulin inhibitors including the napthalene sulfonamides [5, 15, 31], diphenylbutylpiperidines [16] and imidazolines [16] also inhibited the growth of cells. (2) The potency of the drugs as cytotoxic agents correlated with their potency as inhibitors of calmodulin purified from the leukemic cells. This correlation has now been demonstrated with several different classes of inhibitors in the C_6 astrocytoma cell line [16]. Accordingly, melittin was more potent than the phenothiazines. The phenothiazine metabolite, chlorpromazine sulfoxide, was a poor inhibitor of calmodulin and was far less cytotoxic. This latter finding is analogous to those in previous studies that demonstrated that the dechlorinated homolog of the calmodulin inhibitor, *N*-(4-aminobutyl)-5-chloro-2 napthalenesulfonamide, had a decreased affinity for calmodulin [5] and was less cytotoxic in vitro [31] and in vivo [15]. These differences between the parent compound and the metabolites cannot be explained by differences in hydrophobicity, since they have similar octanol-water partition coefficients. The characteristics of the inhibition of growth by these agents are similar to the characteristics of the inhibition of purified calmodulin. Accordingly, the growth-inhibitory effects could not be reversed by excess calcium [11] but could be reversed by excess calmodulin [2], and (4), the enhancement of bleomycin cytoxicity by calmodulin inhibitors could not be demonstrated in *E. coli,* organisms which do not contain measurable calmodulin [6].

The mechanism by which these drugs cause cytotoxicity is under investigation. Calmodulin is essential for many processes that are necessary for normal cellular function, including the assembly and disassembly of microtubules, calcium extrusion from cells by a calcium-magnesium ATPase, and the activation of numerous intracellular enzymes, such as protein kinases, phosphatases, and cyclic nucleotide phosphodiesterase [7, 32]. Interference with any of these known functions of calmodulin would be potentially toxic to cells.

Other cellular functions inhibited by 'calmodulin inhibitors' may or may not be mediated exclusively through the inhibition of calmodulin. These effects include the depletion of intracellular ATP [34], the destabilization of membranes [24] and the inhibition of protein-kinase C.

In summary, the present study demonstrates that inhibitors of calmodulin are cytotoxic to human and murine leukemic cells and that melittin, the potent polypeptide inhibitor of calmodulin, is significantly more cytotoxic than calmodulin inhibitors of other structural classes previously described.

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References

1. Anderson B, Osborn M, Weber K (1978) Specific visualization of the calcium dependent regulatory protein of cyclic nucleotide phosphodiesterase in tissue culture cells by immunofluorescence microscopy: mitosis and intercellular bridge. Cytobiologie 17: 354
2. Boynton AL, Whitfield JF, MacManus JP (1980) Calmodulin stimulates DNA synthesis by rat liver cells. Biochem Biophys Res Commun 95: 745
3. Cadman E, Eiferman F (1979) Mechanism of synergistic cell killing when methotrexate precedes cytosine arabinoside. J Clin Invest 64: 788
4. Chafouleas JG, Pardue GL, Brinkley BR, Dedman JR, Means A (1981) Regulation of intracellular levels of calmodulin and tubulin in normal and transformed cells. Proc Natl Acad Sci USA 78: 996
5. Chafouleas JA, Bolton WE, Hidaka H, Boyd AE, Means AR (1982) Calmodulin and the cell cycle: involvement in regulation of cell cycle progression. Cell 28: 41
6. Chafouleas JG, Bolton WE, Means AR (1984) Potentiation of bleomycin lethality by anti-calmodulin drugs: a role for calmodulin in DNA repair. Science 224: 1346
7. Cheung WY (1982) Calmodulin: an overview. Fed Proc 41: 2253
8. Dedman JR, Welsh MJ, Means AR (1978) Ca^{2+}-dependent regulator: production and characterization of a monospecific antibody. J Biol Chem 253: 7515
9. Durkin JP, Brewer LM, MacManus JP (1983) Occurrence of the tumor-specific calcium-binding protein, oncomodulin, in virally transformed normal rat kidney cells. Cancer Res 43: 5390
10. Ganapathi R, Grabowski D (1983) Enhancement of sensitivity to adriamycin in resistant P388 leukemia by the calmodulin inhibitor, trifluoperazine. Cancer Res 43: 3696
11. Hait WN, Lee GL (1984) Characterization of the cytotoxic effects of calmodulin inhibitors. Clin Res 32: 416A
12. Hait WN, Weiss B (1977) Characteristics of the cyclic nucleotide phosphodiesterase of normal and leukemic lymphocytes. Biochim Biophys Acta 497: 86
13. Hait WN, Cadman E, Benz C, Cole J, Weiss B (1983) Inhibition of growth of L1210 leukemic cells by inhibitors of cyclic nucleotide phosphodiesterase and calmodulin. Proc Am Assoc Cancer Res 2: 50
14. Hickie RA, Wei JW, Blyth LM, Wong DY, Klaassen DJ (1983) Cations and calmodulin in normal and neoplastic cell growth regulation. Can J Biochem Cell Biol 61: 934
15. Ito H, Hidaka H (1983) Antitumor effect of a calmodulin antagonist on the growth of solid sarcoma-180. Cancer Lett 19: 215
16. Lee GL, Hait WN (1985) Growth inhibition of C_6 astrocytoma cells by inhibitions of calmodulin. Life Sciences 36: 347
17. Levin RM, Weiss B (1976) Mechanism by which psychotropic drugs inhibit adenosine cyclic 3′,5′-monophosphate phosphodiesterase of brain. Mol Pharmacol 12: 581
18. Liu YP, Wong VH, Chabner B (1977) Cyclic nucleotide phosphodiesterase and protein activator in human cancer cell lines and Brown-Pearce carcinoma. J Natl Cancer Inst 59: 1605
19. MacManus JP (1975) Occurrence of a low-molecular-weight calcium-binding protein in neoplastic liver. Cancer Res 39: 3000
20. MacManus JP (1981) The stimulation of cyclic nucleotide phosphodiesterase by a M_r 11,500 calcium-binding protein from hepatoma. FEBS Lett 126: 245
21. MacManus JP, Braceland BM, Rixon RH, Whitfield JF, Morris HP (1981) An increase in calmodulin during growth of normal and cancerous liver in vivo. FEBS Lett 133: 99
22. Means AR, Chafouleas JA, Lagace L, Lai E, Stein JP (1982) Multiple roles for calmodulin in the regulation of eukaryotic cell metabolism. In: Gene regulation. Academic, New York, p 307
23. Okumara K, Kato T, Ito J, Tanaka R (1982) Inhibition by calmodulin antagonists of glioblast DNA synthesis and morphological changes induced by glial maturation factor. Dev Brain Res 255: 661
24. Osborn M, Weber K (1980) Damaged cellular function by trifluoperazine, a calmodulin-specific drug. Exp Cell Res 130: 484
25. Prozialek WC, Weiss B (1982) Inhibition of calmodulin by phenothiazines and related drugs: structure-activity relationships. J Pharmacol Exp Ther 222: 509
26. Sellinger-Barnette M, Weiss B (1984) Interaction of various peptides with calmodulin. In: Greengard, Robinson (eds) Advances in cyclic nucleotide and protein phosphorylation research. Raven, New York, p 261
27. Tsuro T, Iida H, Tsukagoshi S, Sakurai Y (1982) Increased accumulation of vincristine and adriamycin in drug-resistant P388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors. Cancer Res 42: 4730
28. Uzunov P, Weiss B (1972) Separation of multiple molecular forms of cyclic adenosine-3′,5′-monophosphate phosphodiesterase in rat cerebellum by polyacrylamide gel electrophoresis. Biochim Biophys Acta 284: 220
29. Watterson DM, Harrelson WG, Keller PM, Sharieb F, Vanaman TC (1976) Calcium-dependent regulatory protein of cyclic nucleotide metabolism in normal and transformed chicken embryo fibroblasts. J Biol Chem 25: 4501
30. Wei J-W, Morris HP, Hickie RA (1982) Positive correlation between calmodulin content and hepatoma growth rates. Cancer Res 42: 2571
31. Wei J-W, Hickie RA, Klaassen DJ (1983) Inhibition of human breast cancer colony formation by anticalmodulin agents, trifluoperazine, W-7 and W-13. Cancer Chemother Pharmacol 11: 86
32. Weiss B, Wallace TL (1980) Mechanism and pharmacological implications of altering calmodulin activity. In: Cheung WY (ed) Calcium and cell function, vol 1. Academic, New York, p 329
33. Willingham MC, Wehland J, Klee CB, Rickert ND, Rutherford AV, Pastan IH (1983) Ultrastructural immunocytochemical localization of calmodulin in cultured cells. J Histochem Cytochem 31: 445
34. Zimmerman TP, Wolberg G (1984) Effects of calmodulin antagonists on lymphocyte-mediated cytolysis. Proc Am Assoc Cancer Res 25: 351

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