Targeting Calmodulin in Reversing Multi Drug Resistance in Cancer Cells

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Abstract: Calmodulin is a Ca^{2+} binding protein found in many eukaryotic cells. It is one of the most important intracellular mediators of Ca^{2+}-dependant signaling in eukaryotic cells. It regulates diverse processes including mitosis, muscle contraction and nucleotide metabolism by modulating the activity of at least 30 different target enzymes in a calcium-dependant manner. Calmodulin plays an important role in the regulation of processes, such as the assembly and disassembly of microtubules by controlling protein kinase activities, by exerting an indirect influence upon a wide variety of cellular processes. It is observed that multi-drug resistant cells have a greater intracellular concentration of calcium than non-resistant cells which contributes to their increased sensitivity to calmodulin antagonism compared with that of non resistant cells. Calmodulin mediated processes can be effectively inhibited by a variety of pharmacological agents of different chemical structures, eg:The calcium channel blocker verapamil and antipsychotic drugs like the Phenothiazines. Many bioisosteres of phenothiazines like phenoxazines and acridones have been prepared and these have also shown very good calmodulin antagonism. These calmodulin antagonists have been shown to modulate multi-drug resistance (MDR) in cancer cells. This review highlights concepts of identification and optimization of new inhibitors of calmodulin in reversing MDR in cancer cells.

Key Words: Multi drug resistance, calmodulin, phosphodiesterase, P-glycoprotein.

INTRODUCTION TO CALMODULIN

Calcium ion is widely recognized as a major regulator of intracellular metabolism throughout the animal kingdom. Calcium appears to exert its biological effects as a secondary messenger or signal transducer through its interaction with calcium-modulated proteins. Through the efforts of many research workers over the past two decades, a number of Ca^{2+}-binding proteins have been characterized as having properties, to play the role as putative intracellular Ca^{2+} receptors. These proteins include the variant forms of troponin C from cardiac and skeletal muscle, parvalbumin, a vitamin D inducible protein, calcineurin, calsequestrin and calmodulin. Binding of calmodulin to particulate fractions has been reported to require Ca^{2+} and appears to occur at specific sites [1,2]. Earlier studies have shown that a generalized calcium target protein, called calmodulin, exists in eukaryotic cells and regulates a number of enzyme systems in response to calcium flux. Calmodulin was first described by Cheung [3], as an activator of cyclic nucleotide phosphodiesterase. During the same period, Kakiuchi *et al*. [4] reported the presence of calmodulin in the brain extracts of a phosphodiesterase-activating factor specific for the Ca^{2+}-dependant phosphodiesterase.

Teo and Wang [5] later demonstrated the identity of two proteins and the Ca^{2+}-binding property of calmodulin. It is a small, heat- and acid- stable protein whose amino acid sequence has been conserved throughout evolution. The molecular weight of the protein based on its amino acid sequence is 16,790 Da. [6]. The protein exists as a monomer and it is a highly acidic protein (isoelectric point 3.9-4.3). Calmodulin concentration and location play an important role in regulating its biological activity. Calmodulin constitutes at least 0.1% of the total protein present in cells (10^{-6}-10^{-5} M) and is expressed at even higher level in rapidly growing cells, especially those undergoing cell division and differentiation [7].

Calmodulin is widely distributed in the tissues of eukaryotes. By indirect immunofluorescence techniques, calmodulin has been shown to be generally distributed throughout the cytoplasm of interphase proliferating cells in tissue culture [8,9]. During mitosis, much of the protein appears to associate with the mitotic apparatus especially around the centrioles and the cytoplasmic furrow during cytokineses [10]. Another study conducted with tissue slices of rat liver has shown that calmodulin is associated with cytoplasm, nucleus, plasma membrane and glycogen particles [11].

The protein mediated the control of a large number of enzymes by Ca^{2+}. Among the enzymes so far known to be stimulated by the calmodulin Ca^{2+} complex are cyclic nucleotide phosphodiesterase, brain adenylate cyclase, the ATPase and Ca^{2+} pump of the erythrocyte plasma membrane, myosin light chain kinases, brain membrane kinases, phosphorylate b kinase and NAD kinase in plants. Cheung reported that the calmodulin and phosphodiesterse form a stoichiometric complex during activation [12]. Calmodulin also plays an important role in the regulation of other processes, such as the assembly and disassembly of microtubules and by control of protein kinase activities, which may exert an indirect influence upon a wide variety of cellular processes. The presence of four similar but distinguishable Ca^{2+}-

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binding domains in calmodulin allows the possibility that different proteins may interact with different regions of the calmodulin Ca^{2+}complex. Much may be known by the careful study of the 16 possible calmodulin Ca^{2+} complexes and their interaction with different enzymes. Calmodulin possesses the property of conferring reversible activation upon a number of enzymes and its ability to serve as a multifunctional Ca^{2+}-dependant regulator, and its physical interactions with a group of different enzymes, may be unique among regulatory proteins. It is this ability of calmodulin that has aroused the current widespread interest to study its biochemical and pharmacological aspects.

ROLE OF CALMODULIN IN CANCER

Ca^{2+}-dependant signaling plays a very important role in cellular metabolism. Alteration in any of the components of signaling pathway could lead to states of abnormal cellular proliferation. There are evidences to show that calmodulin plays an important role in both normal and abnormal states of cellular proliferation [13,14]. The evidence suggests that this permissive effect is mediated through calmodulin remains to be proven. Increasing concentration of calcium in growth medium from 0.02 to 1.25 μM initiates DNA synthesis in T51 rat hepatocytes [15]. The initiation of DNA synthesis by calcium was mimicked by calmodulin and blocked by a calmodulin antagonist and an anti-calmodulin antibody. Furthermore, calmodulin has been shown to be involved in microtubular assembly and disassembly [16] and vincristine (Fig. (**1**)) a drug that inhibits microtubular assembly, is also shown to inhibit calmodulin stimulated enzymatic activity [17]. Also, it has been shown that the intracellular concentration of calmodulin changes as cells go through the cell cycle [18, 19]. The concentration of calmodulin is increased in proliferating cells as compared with non-proliferating cells. The increased concentration of Ca^{2+} in the rapidly proliferating cells may result in a greater proportion of activated calmodulin and a greater stimulation of calmodulin mediated process. A change in the molecule or its function(s) may result in states of abnormal cellular replication. There are numerous ways in which the calmodulin branch of the calcium messenger system may be altered in malignancy for example an alteration in the quantity of cellular calmodulin. This has been shown in some studies on malignant tissues and transformed cell lines [13,14]. The result of an increased concentration of intracellular calmodulin in malignancy alters the interaction of calmodulin with its target proteins. Calmodulin is highly conserved throughout the phylogenetic spectrum. There is evidence of altered forms or production of defective calmodulin [19]. This has occurred in both mammalian species [20] as well as non-mammalian species

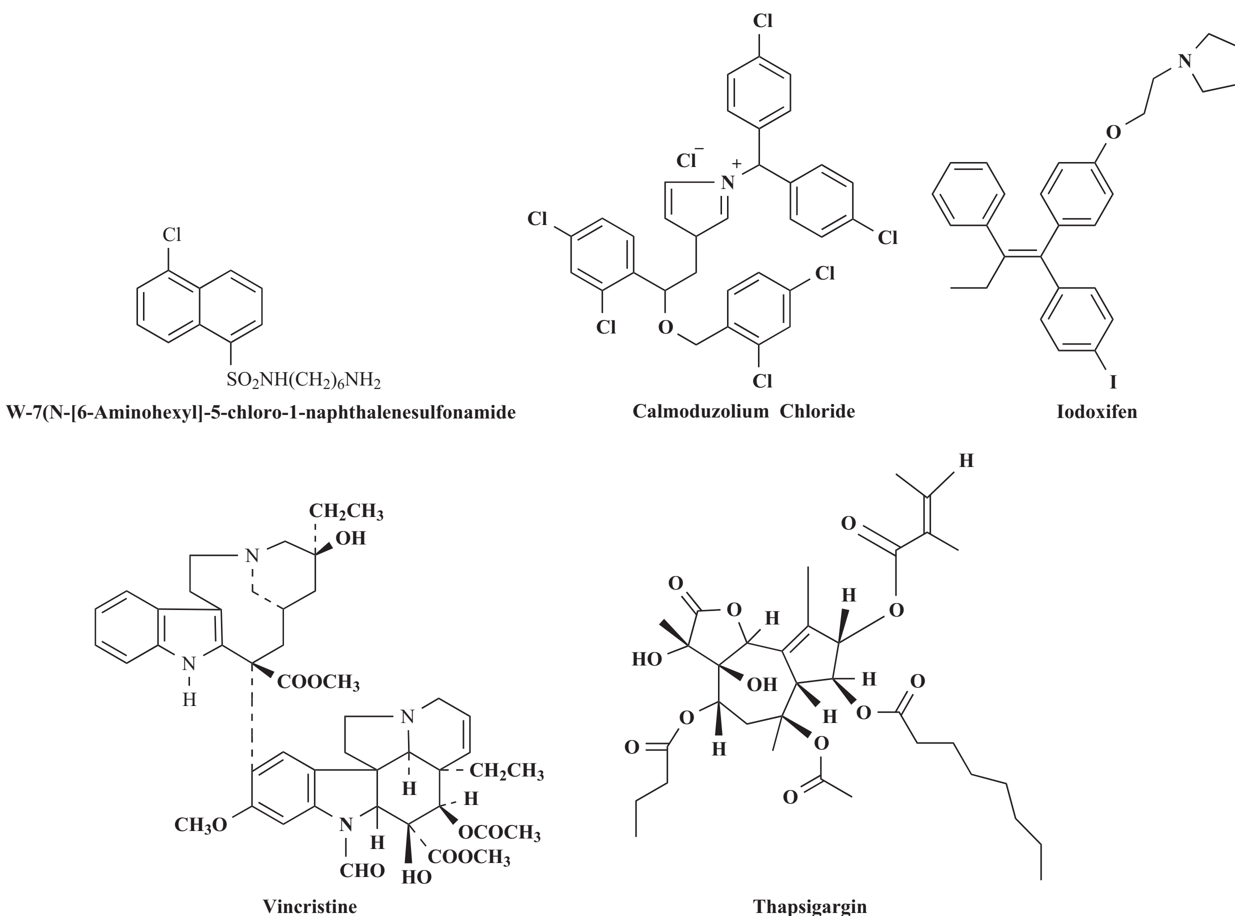

Fig. (1). Structures of calcium channel blockers with chemosensitizing activity.

such as trypanosome [21] in which marked structural and functional differences in the calmodulin molecule have been identified. Fukaumin *et al*. [22] have demonstrated that the tyrosine residues of calmodulin are phosphorylated in the cells transformed by the *Rous sarcoma* virus. Since the two-tyrosine residues of calmodulin are both located within the calcium-binding domain, it has been suggested that this could lead to an alteration in the structure and function of calmodulin.

MacMannus and co-workers [23] isolated a calmodulin-like protein, which they called "oncomodulin" from malignant cells. This protein most closely resembled the parvalbumin family of calcium-binding proteins and shared structural and physiological features with calmodulin. It was also found to be an acidic and calcium-binding protein that selectively activated calmodulin sensitive *phosphodiesterase* (PDE) from the heart but did not activate the PDE enzyme from the brain [24]. Alterations of the calmodulin's target proteins in malignant cells could change the function of calmodulin in malignant state. Since calcium saturated calmodulin is more sensitive to inhibition by most calmodulin antagonists, it is speculated that in certain instances, malignant cells may be more sensitive to inhibition by drugs than their normal counterparts. The observation that multi-drug resistant cells have a greater intracellular concentration of calcium than non-resistant cells may contribute to their increased sensitivity to calmodulin antagonists compared to that of sensitive cells [25].

Recent studies have indicated that calmodulin has affinity for the estrogen receptor (ER) [26,27]. It has been reported that there is a direct physical interaction between purified calmodulin and ER. This direct interaction may be an initial event preceding the assembly of ER plus auxiliary proteins into the active ER complex with its DNA motif, the estrogen response element. Calmodulin is functionally required for activation of an ER-responsive promotor, in the 17β-estradiol-ER pathway of hormonal action and regulation of 17β-estradiol-responsive gene expression that is associated with proliferation of mammary epithelial cells. Iodoxifen is a novel selective estrogen (E2) receptor modulator that is currently in clinical development for the treatment of breast cancer. Stephen *et al*. [28] have reported that iodoxifen has affinity towards ER and reduced agonistic activity against breast and uterine cells. Further, they also demonstrated the anticalmodulin activity of iodoxifen (Fig. (**1**)).

Another group while studying the functional role of the molecules demonstrated that a pertinent system is involved in signal transduction pathways and mechanism of metastasis and invasion of colon carcinoma cells [29]. The prominent molecule, among the members involved in these signal transduction pathways was found to be calmodulin. They have shown that the calmodulin inhibitors (W-7 and calmidazolium Fig. (**1**) are the ones that inhibit cell adhesion. Tombal *et al*. [30] have demonstrated the ability of the new molecule, Thapsigargin (TG) (Fig. (**1**)) to perturb intracellular free Ca^{2+} and its induction of a calmodulin/calcineurin-dependent apoptotic cascade responsible for the death of prostatic cancer cells, resulting in DNA and cellular fragmentation in apoptotic bodies. Tumor cell resistance to inhibitors of topoisomerase II is often associated with the over expression of P-glycoprotein (P-gp) [31]. Inhibitors of calcium-calmodulin-dependent enzyme are shown to sensitize tumor cells to the topoisomerase II poison etoposide (vp-16) by enhancing DNA damage and an apoptotic response. Grabowski *et al*. [31] have shown that intracellular calcium transients could play a key role in the sensitization of etoposide-resistant tumor cells by inhibitors of calcium calmodulin-dependent enzyme.

CALMODULIN ANTAGONISTS AS ANTI-CANCER AGENTS

Calmodulin plays an important role in regulating many physiological processes. Such fundamental biochemical regulator may provide a route for important pharmacological intervention. The identification of drugs that alter the activity of calmodulin may therefore, provide a new approach to altering physiological or pathological processes. Calmodulin mediated processes can be effectively inhibited by a number of pharmacological agents of widely different chemical structures. Knowledge of structural details of their interaction with calmodulin is crucial in understanding the molecular mechanism(s) of action. Therefore it is very essential to determine the structural basis for the interaction of Ca^{2+} and calmodulin with drugs, vincristine, phenothiazine derivatives and verapamil, of various chemical structures. This knowledge has to be used to elucidate molecular mechanisms of action of calmodulin binding drugs. Due to the central role of calmodulin in propagating the calcium signal towards different cellular processes, drugs which modulate calmodulin action offer tools for dissecting and investigating calmodulin dependent pathways. The various classes of drugs differ greatly in the potency as inhibitors of calmodulin. Although the compounds having anti-calmodulin activity have been catalogued into different pharmacological classes, they share many common structural features like hydrophobic ring, a side chain, and a secondary amine and display considerable overlap in their pharmacological actions like anti-hypertensive, antipsychotic, anti-cancer and anti-MDR activities.

Phenothiazines and structurally related antipsycotics inhibit the activity of several cellular enzymes [32, 34, 35] and block the function of critical cellular receptors such as those for dopamine [36]. Among these cellular targets calmodulin, the functional calcium binding protein, [37] has been implicated in their regulation of numerous cellular events, including that of normal and abnormal cellular proliferation [13,38,39]. Consistent with these observations was the demonstration that phenothiazines and other calmodulin antagonist possess antiproliferative and cytotoxic effects [40] that were proportional to their anti-calmodulin activity [41-44]. Certain drugs inhibit the calmodulin-induced activation of particular forms of *phosphodiesterase*. The mechanism, by which these drugs act by binding directly to calmodulin in reversible, calcium-dependent manner, may provide a common mechanism for explaining some of the diverse biochemical actions of these drugs. This event has lent support to the hypothesis that several of the apparently unrelated biochemical effects of these drugs may be due to common mechanism of binding to and inhibiting calmodulin.

CALMODULIN ANTAGONISTS AS REVERSERS OF MDR IN CANCER CELLS

Results from different laboratories indicate that calmodulin antagonists can be exploited for therapeutic benefit, as they enhance the cytotoxic and cytostatic effects of doxorubicin, vincrinstine and bleomycin. The recent demonstration and elucidation of the phenomenon of MDR have lead to the search for drugs that could sensitize highly resistant cancer cells to chemotherapeutic agents. MDR is a process where by malignant cells become resistant to structurally diverse chemotherapeutic agents following exposure to a single type of cytotoxic drug [45]. Certain MDR cell lines have been associated with a decrease of drug accumulation due to enhanced efflux of chemotherapeutic drugs [46]. These effects have been attributed to the over expression of the 170 kDa membrane glycoprotein (P-gp), which structurally resembles transport proteins in prokaryotic cells [47] and may function as an energy dependant drug efflux pump in mammalian cells [48,49].

Modulators came after the observations that calcium channel antagonist verapamil [50] (Fig. (**2**)) and calmodulin antagonist [51] reversed drug resistance. Phenothiazines [52] (Fig. (**3**)) and phenoxazines [53] (Fig. (**4**)) have been shown to be among the group of the drugs to modify MDR. Although the mechanism by which phenothiazines, phenoxazines, and other drugs that modulate MDR is not clear, it has been suggested that their pharmacological action may be mediated by the calcium messenger system, because the active compounds are known to inhibit voltage dependent calcium channels [54], Calmodulin [55] and protein kinase C [56]. Mechanism by which modulators exert their anti-MDR action remain obscure. However it can be speculated that alteration in P-gp primary structure or post translational modification of P-gp may be involved, particularly when a modulator may have pharmacological effects like calmodulin or protein kinase C inhibition, which could influence the functional state of P-gp. Another aspect, yet to be established in the study of MDR modulation, is to ascertain whether there is any interaction between calmodulin with P-gp or not.

One aspect generally over looked in the study of MDR modulation is interaction of modulators with the lipid domains surrounding P-gp. If modulators alter lipid composition of the plasma membrane the change could explain the observed increase in drug permeability [57]. Kessel and Wilverding [58] have suggested that accumulation of promoters such as calmodulin antagonist may act by membrane modifications resulting in impairment of outward transport. It has been observed that inhibition of calcium binding to calmodulin has also been related to hydrophobic interactions [59] and drug induced perturbations [60].

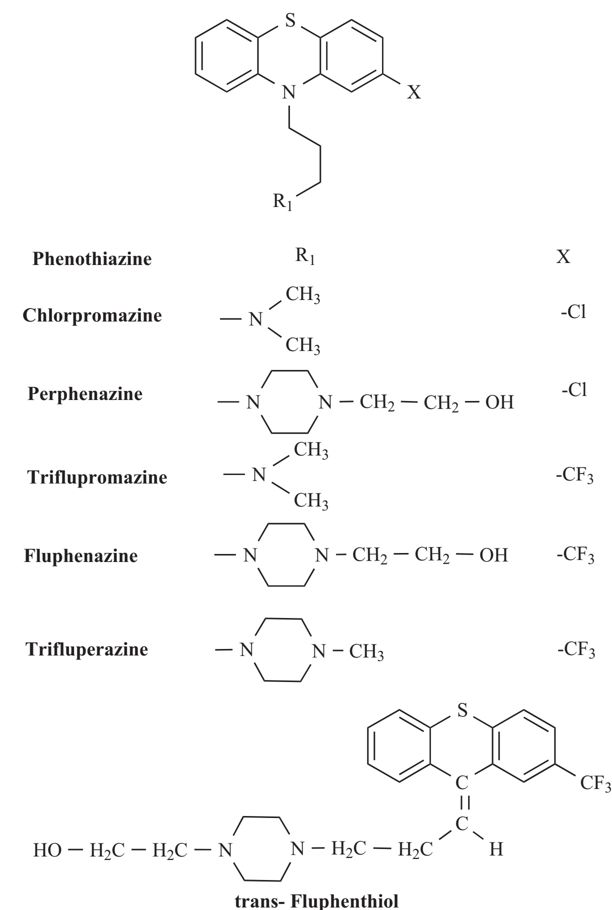

Fig (3). Structures of phenothiazines and thioxanthenes with chemosensitizing activity.

Tsuruo *et al.* [51] first described the MDR reversing properties of phenothiazine agents whose primary four to five fold increase activities were directed against calmodulin mediated processes. Non-toxic concentrations of trifluoperazine caused a four to five fold increase in accumulation of vincristine and doxorubicine and potentiated the activity of these agents in vincristine and doxorubicine resistant P388 cells.

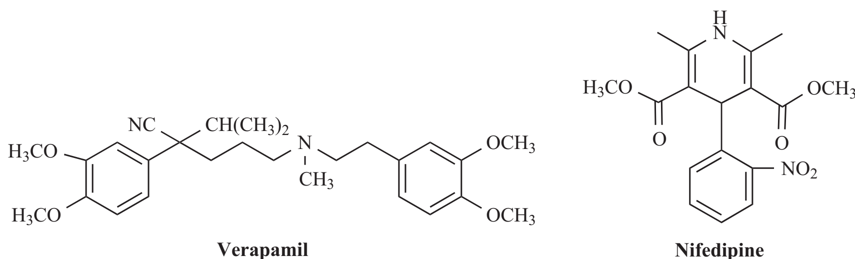

Fig. (2). Structures of calcium channel blockers with chemosensitizing activity.

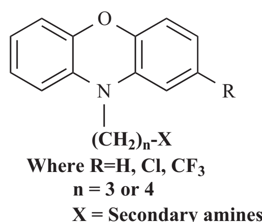

Fig. (4). Structure of phenoxazines with different substitutions.

Ford *et al.* [61] examined the structure activity relationships of series of 22 phenothiazines for reversing doxorubicin resistance in a 220 fold resistant MCF-7^{Adr} cell line. They found that hydrophobicity of tricyclic ring and structural features of the N^{10} and the substituent amine possessed the greatest activity which was further enhanced by substitution at C2 position. Prochlorperazine, fluphenazine and trifluperazine fulfilled these structural requirements, but were relatively poor reversing agents causing at most of 3-fold potentiation of doxorubicin potency. More studies attempting to understand the structure- activity relationship for phenoxazines have been reported by Thimmaiah *et al.* [62-64]. In an initial study, the role of the electronegativity of phenothiazine nucleus was examined. It was found that phenothiazines increased accumulation of vincristine and vinblastine 2-fold, whereas phenoxazine having an oxygen atom in place of sulphur in tricyclic ring was significantly more effective than verapamil in increasing the accumulation of vinca alkaloids and was less effective in sensitizing the MDR cells. Jagadeesh S (Ph.D. Thesis, Department of Chemistry, University of Mysore, India 2001) [65], made an effort to identify more active phenoxazine molecules and the effect of a series of phenoxazine molecules on calmodulin activation of PDE was studied. The results show the structure and effect on calmodulin, of a series of phenoxazines having different substitutions on the phenoxazine nucleus. The IC_{50} values for inhibition of calmodulin by parent molecules like phenoxazine, 2-chlorophenoxazine (substitution of a -Cl at position 2), 2-trifluorophenoxazine (substitution of a $-CF_3$ at position 2), were more than 100 μM. It was difficult to increase the concentration to more than 100 μM because of the solubility problem. Substitution of –Cl at position 2 increased the inhibitory activity compared with unsubstituted phenoxazines. Compared with –Cl substitution, substitution of $-CF_3$ at position 2 increased the anti-calmodulin activity. A comparative study of the three series of phenoxazine compounds has revealed that the anticalmodulin activity largely followed the order: 2-trifluoromethyl-N^{10}-> 2-chloro-N-^{10}-> N^{10}-substituted phenoxazines.

Similarly Hegde *et al.* [66] have studied the inhibition of calmodulin activity and their studies revealed that 4-methoxyacridones (Fig. (**5**)) exhibited slightly higher potency than the unsubstituted counterparts. Comparison of the IC_{50} values within the series revealed that the butyl derivatives are found to possess greater inhibitory potency than the propyl derivatives. The most potent acridone derivatives found against the calmodulin activation of *c*AMP-phosphodiesterase were the analogues suggesting that the variation of the side chain is a strict requirement for activity. Replacement of bishydroxyethylamino or β-hydroxyethylpiperazino

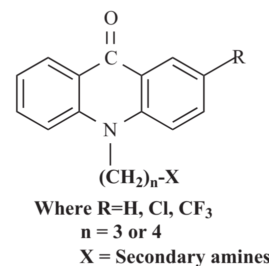

Fig. (5). Structure of N^{10}- substituted acridone derivatives.

substituent by –Cl reduced the activity and introduction of a –H atom abolished it. The results showed a strong positive correlation between the inhibition of calmodulin-dependent cyclic AMP-phosphodiesterase activities and cytotoxicities or anti-MDR activity. Through the use of acridone derivatives, we have shown a good correlation between inhibition of calmodulin and *in vitro* cytotoxicities or anti-MDR activity [65,66]. These results suggest that inhibition of calmodulin, an intracellular calcium-binding protein that is known to play a key role in regulating cell proliferation, may be of importance in mediating the cytotoxic effects of acridones.

Recently Mayur *et al.* [67], have studied the anti-calmodulin activity of different 2-bromo-N^{10}- substituted acridones. Comparison of the IC_{50} values within the series revealed that the butyl derivatives are found to possess greater inhibitory potency than the propyl derivatives. A comparative study of a series of acridones differing with respect to amino substitution on the side chain showed that anticalmodulin activity largely followedbishydroxyethyl piperazino > diethanolamino > morpholino > piperidino. The results showed IC_{50} values for the inhibition of cell growth were related to their effect on MDR. A good correlation was found between anti-calmodulin, anti-proliferative (r = 0.9309) and reversal of MDR (r = 0.7115) for KBCh^R-8-5 cells.

STRUCTURAL REQUIREMENT FOR ANTI-CALMODULIN ANTAGONISTS AS ANTI-MDR AGENT

From the above study, the preliminary assessment was that the necessary structure requirements for a modulator to be potent inhibitor of calmodulin in reversing MDR in cancer cells would be:

1. Large hydrophobic region, consisting tricyclic rings.
2. A substitution at 2-position (preferably) to increase hydrophobicity.
3. Alkyl side-chain should contain at least three carbon atoms.
4. Highly charged amino group (two positive charged nitrogens as in β-hydroxy piperzinyl) at the end of alkyl bridge.

CONCLUSIONS

The importance of calmodulin in the regulation of cellular processes such as mobility, secretion, division, cell shape and metabolic activity is well established. A wealth of literature continues to support the conclusion that calmodulin is a multifunctional, Ca^{2+}-dependent enzyme regulator. Its prop-

erties and distribution are in accordance with its function as a major, if not the predominant, Ca^{2+} receptor protein in most cell types. Calmodulin acting in this capacity would couple transient increases in intracellular free Ca^{2+} resulting from extracellular stimuli to the activation of rate-limiting enzymes in diverse metabolic processes. In this capacity it would co-ordinate the processes involved in maintaining the functional status of cell.

Antagonism of calmodulin has been shown to correlate closely with inhibition of growth of malignant cell lines because it plays a central role in processes concerned with normal cellular viability. Hence, alteration in calmodulin may contribute to the loss of viability of malignant cells. The studies showed an excellent correlation between the ability of drugs to block the activation of phosphodiesterase and their ability to bind to the activator and showed a good qualitative relationship between anti-proliferative activity and binding to the activator.

These results support the proposal that the mechanism by which modulators inhibit cell proliferation may be direct binding to activator. Moreover, the evidence that calcium-dependent protein can activate several other enzyme systems, each of which is inhibited by the acridone anti MDR-modulator, has suggested that the binding of acridones to activator may be the mechanism by which these modulators exert several of their biochemical and pharmacological actions. Hiroyoshi *et al.* [62] have shown that W-7calmodulin–antagonist arrests the growth of the cells at the G1/S boundary phase of the cell cycle. They also demonstrated that the initiation of DNA synthesis required Ca^{2+} and calmodulin. However, cell proliferation is a complex phenomenon involving several systems. It is likely that the entire range of pharmacological effects of calmodulin antagonists on cancer cell proliferation results from a combination of several types of molecular interaction and cannot be simply be explained by a single mechanism.

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