Sensitization of Multidrug Resistant (MDR) Cancer Cells to Vinblastine by Novel Acridones: Correlation between Anti-Calmodulin Activity and Anti-MDR Activity

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Abstract: Multidrug resistance (MDR) of cancer cells remains to be an important cause of chemotherapy failure. Search for the new MDR reversal agents is still an unceasing challenge for the scientists. In an attempt to find clinically useful modulators of MDR, a series of 19 N^{10}-substituted-2-bromoacridones has been synthesized. Parent compound 1, prepared by the Ullmann condensation of *o*-chlorobenzoic acid and *p*-bromoaniline, undergoes *N*-alkylation in the presence of a phase transfer catalyst. *N*-(ω-Chloroalkyl) analogues were subjected to iodide catalyzed nucleophilic substitution reaction with various secondary amines to get the products **3-10** and **12-19**, which increased the uptake of vinblastine (VLB) in MDR $KBCh^R$-8-5 cells to a greater extent (1.25 to 1.9-fold) than did a similar concentration of the standard modulator, verapamil (VRP). Results of the efflux experiment showed that each modulator significantly inhibited the efflux of VLB, suggesting that they may be competitors for P-gp. All the compounds effectively compete with [^{3}H] azidopine for binding to P-gp, pointed out this transport membrane protein as their likely site of action. Compounds at IC_{10} were evaluated for their efficacy to modulate the cytotoxicity of VLB in $KBCh^R$-8-5 cells and found that the modulators enhanced the cytotoxicity of VLB by 3.8 to 34-fold. The study on the structure-activity relationship revealed that substitution of hydrogen atom at position C-2 in acridone nucleus by a bromine atom increased the cytotoxic and anti-MDR activities. The ability of acridones to inhibit calmodulin-dependent cyclic AMP phosphodiesterase has been determined and the results have shown a strong positive correlation between anti-calmodulin activity and cytotoxicity in $KBCh^R$-8-5 cells or anti-MDR activity.

Key Words: 2-Bromoacridones, anti-MDR activity, anti-calmodulin activity, structure activity relationships, correlation study.

INTRODUCTION

Clinical resistance to drugs is a major obstacle in cancer chemotherapy today. Although there are many antineoplastic drugs in use, only a few are effective in the treatment of each specific tumor type because of intrinsic or acquired drug resistance. A broad-spectrum resistance to structurally and mechanistically diverse antitumor agents is known as multidrug resistance (MDR) [1]. Classical MDR results from the overexpression of ATP-binding cassette (ABC) transporters [2]. The two best-known and extensively studied ABC transporters are P-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1) [3]. A large number of structurally unrelated compounds are known to be P-gp substrates, only having in common high hydrophobicity, an amphiphilic nature, and a net positive charge [4]. MRP1 also effluxes a broad range of substrates, either by glutathione co-transport mechanism or after their conjugation to glutathione [5,6]. P-gp and MRP1 are typically coexpressed with other ABC transporters such as breast cancer resistance protein (BCRP) [7-11]. Recent work has also highlighted the importance of receptor kinases and cell survival/anti-apoptotic pathways in drug resistance [12-16] but very few connections have been made between the latter pathways and multidrug transporter expression or function. However, a recent study has shown that the PI3kinase/Akt pathway influences expression of the MRP1 transporter in prostate carcinoma cells [17]. Other approaches tested application of antisense oligonucleotides [18-21] and ribozymes [22] to reduce P-gp effects for an enhanced efficacy of cancer treatment.

As soon as P-gp and sister proteins were recognized to be responsible for MDR, blocking the efflux of drugs by inhibiting the functions of these transporters became a realistic way to overcome MDR [23]. The use of noncytotoxic chemosensitizers (MDR-reversal agents, MDR modulators) which can block the anticancer drug binding sites, thus preventing their exclusion from the cells, has received considerable attention to date. Tsuruo and co-workers [24] were the first to demonstrate the ability of the calcium channel blocker, VRP, to reverse MDR. Since then, many

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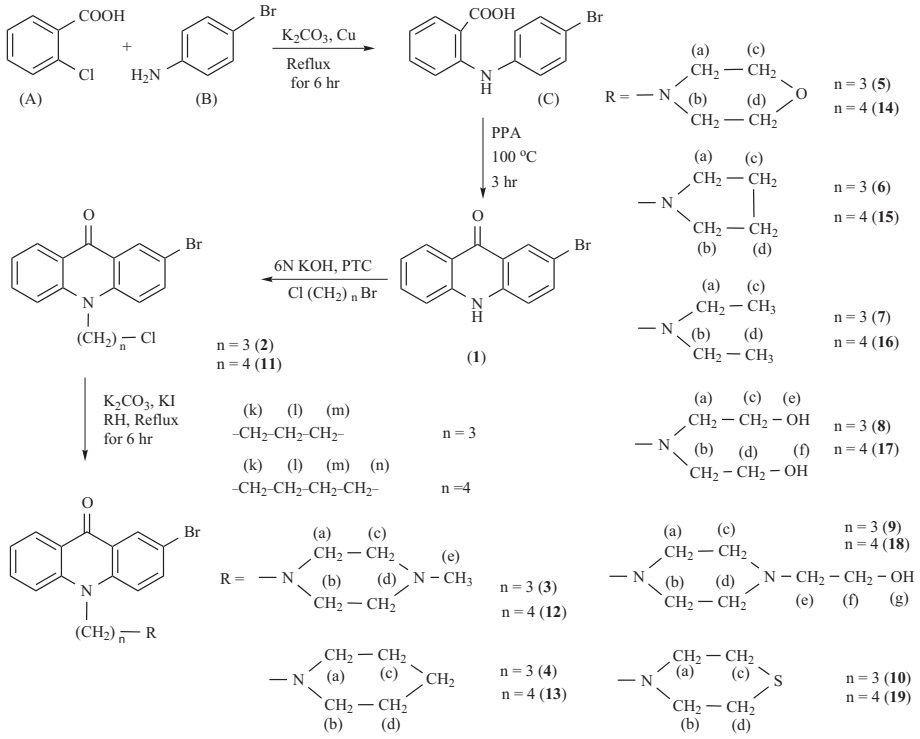
other reversal agents were brought to light, such as calmodulin antagonists (trifluoperazine), antiarrhythmics (amiodarone), antihypertensive agents (reserpine), antipsychotics (phenothiazines), and immunosuppressants (cyclosporine A, FK506) [25-29]. However, undesirable side effects limited their use in clinical trials. Thus, potent noncytotoxic reversal agents with minimum undesirable side effects are in demand.

Most therapeutic approaches to overcome MDR are mainly aimed at coadministration of drugs that bind to P-gp and block its excretory function. The majority of the previous studies in our laboratories as well as others have focused on specific MDR inhibitors, only targeting P-gp, despite the fact that additional ABC transport proteins contribute to clinical MDR. It has been shown that P-gp and BCRP are significantly coexpressed, and P-gp expressed with MRP1 greatly increases the efflux of chemotherapeutic agents [7-11]. Therefore, the design of potent MDR inhibitors devoid of other pharmacological activities has thus become a desirable goal to test the MDR reversal hypothesis in the clinic. In our program for the development of new anti-MDR agents, we demonstrated a novel acridone derivative, [1,3-bis (9-oxoacridin-10-yl]propane, as a potent and poorly reversible modulator of Pgp-mediated VLB transport [30]. Subsequently, the authors prepared the parent 2-bromo, 2-methoxy and 4-methoxy acridones and evaluated for anti-MDR activity against $KBCh^R$-8-5 cells. Examination of the results revealed that a bromo- group at C-2 position exhibited the highest anti-MDR activity as compared to methoxy substituent at C-2 or C-4 position [31, 32]. Therefore, to extend the portfolio of better transporter antagonists, the authors describe the synthesis and biological evaluation of a series of nineteen 2-bromo-N^{10}-substituted acridone derivatives as potential MDR reversing agents for use in cancer chemotherapy. Furthermore, to clarify the role of calmodulin in these processes, we have compared the anti-calmodulin effects of some of these potent acridones with their effects on cellular growth and P-gp-mediated MDR.

CHEMISTRY

Acridones, which belong to an important group of heterocyclic compounds, due to their exceptional diverse activity, attracted the scientific community and have been emerging as a new class of bioactive molecules. The ease of synthesis, attractive coloration, and crystallinity of acridine compounds have attracted the attention of the chemists [33]. The basic tricyclic framework can be decorated with suitable substituents to confer specificity against both prokaryotic and eukaryotic targets which have given acridines a respectable reputation in the history of chemotherapy [34]. There are several examples of acridine and acridone derivatives with or without an alkyl side chain attached to the N^{10}-position, with diversified biological activities [35-38]. As part of our research efforts towards the synthesis of anti-MDR agents, the authors have focused on developing simple synthetic routes together with improvements over published methods to give the first account of anti-MDR activity of 2-bromoacridone derivatives .

2-Bromoacridone (**1**) and its derivatives (**2-19**) were prepared by the synthetic route as outlined in Scheme (**1**). The general procedure for preparing *N*-alkyl derivative



Scheme 1.

consists of condensation of acridone with requisite amount of alkyl halide in the presence of a strong acid binding agent like sodium amide in anhydrous aromatic solvents such as toluene or benzene. The reaction of 2-bromoacridone with chlorobromoalkanes in the presence of sodium amide in anhydrous toluene under reflux conditions gave respective N^{10}-(chloroalkyl) acridone. But, the yield was very low, besides requiring drastic experimental conditions. Acridone has a less basic nitrogen atom and the previously described preparative procedure for *N*-alkylation needs sodamide besides using anhydrous aromatic solvent under reflux conditions. However, this compound undergoes *N*-alkylation in the presence of a phase transfer catalyst (PTC) more easily compared to previously described preparative procedure.

Stirring of compound (**1**) at room temperature with alkylating agent Br-$(CH_2)_3$-Cl or Br-$(CH_2)_4$-Cl in a two phase system consisting of an organic solvent (tetrahydrofuran) and a 6 N aqueous potassium hydroxide solution in the presence of tetrabutylammonium bromide [$(n\text{-}C_4H_9)_4N^+Br^-$] leads to the formation of the respective 10-(3'-chloropropyl) (**2**) or 10-(4'-chlorobutyl) (**11**) acridone in good yield.

Iodide catalyzed nucleophilic substitution of the N^{10}-chloropropyl or N^{10}-chlorobutyl acridone with various secondary amines (*N*-methylpiperazine, piperidine, morpholine, pyrrolidine, diethylamine, diethanolamine, thiomorpholine and (β-hydroxyethyl) piperazine) by refluxing for different time intervals in the presence of potassium carbonate in acetonitrile gave the free bases (**3-10,** and **12-19**).

All the products were separated and purified by column chromatography or recrystallization method and dried under high vacuum for more than 12 hours. The purified compounds were characterized by UV, IR, ^{1}H- and ^{13}C-NMR and mass spectral methods. The UV spectral data of acridones are in close agreement with the spectral characteristics of analogous heterocyclic compounds [39, 40]. The characteristic bands in the IR spectra support the structure of acridones. The assignment of protons is fully supported by the integration curves and all the derivatives showed the characteristic chemical shifts for the acridone nucleus. The assignment of the ^{13}C-resonances of acridone derivatives are in close agreement with an analogous compound N^{10}-alkyl substituted 9(10)-acridone.

PHARMACOLOGICAL ACTIVITY

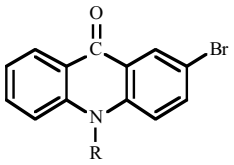
pK_a and Lipophilicity Effects

The acridone derivatives are weak bases and able to exist in both protonated and unprotonated forms. The unprotonated or neutral form of compounds will be highly membrane permeable and able to diffuse freely and rapidly across biological membranes. In contrast, the protonated form would be at least an order of magnitude less membrane permeable and diffuse across membranes at a much reduced rate. In addition, if the unprotonated form of the molecule diffuses across the membrane and enters an acidic compartment within the cell, it will rapidly become protonated and unable to diffuse out of the cell. The magnitude of the biological activity depends on pK_a of compounds besides other factors. For the series of compounds examined, the pK_a values (Table **1**) ranging from 8.35 - 9.51 lie closer to physiological pH, which may suggest that these compounds accumulate in MDR cells as free bases rather than in protonated form. The lipophilicity data varying from 1.40 - 2.11, expressed in $\log_{10}$P for nineteen acridone derivatives are given in Table **1**. Within the series, all the compounds were highly lipophilic at pH 7.4 and it is expected that they will accumulate rapidly into the cells. Analysis of the relationship between lipid solubility ($\log_{10}$P) and the effectiveness of the modulators to increase VLB accumulation in drug-resistant $KBCh^R$-8-5 cells showed a reasonably good correlation ("Fig. (**1**)", $R^2 = 0.50$).

Effect of Acridone Derivatives on Cellular Accumulation and the Efflux of [^{3}H]VLB

To identify the possible mechanism of potentiation of VLB by acridones, the effect of these compounds at their IC_{50} concentrations (Table **3**) on the cellular accumulation of VLB in P-gp mediated MDR $KBCh^R$-8-5 cells has been investigated and the results are given in Table **1**. All the compounds tested, exhibited a significant enhancing effect on VLB accumulation relative to control. As shown in Table **1**, compounds exhibited significant VLB accumulation enhancing effect (7 to 21-fold relative to control) compared to a standard modulator VRP (11.9-fold). Nine compounds (**1-4, 6-8, 10** and **15**) were found to possess less VLB enhancing effect (7.0 to 11.5- fold) compared to VRP (11.9-fold) relative to control. Remaining ten compounds (**5, 9, 11-14,** and **16-19**) caused a 1.3 to 1.9-fold greater accumulation of VLB than did a similar concentration of VRP. The enhancement of VLB uptake was specific for MDR cell line since all compounds had very little effect in sensitive KB-3-1 cells. Comparative study of the VLB uptake data of the modulators within the propyl derivatives revealed that all the propyl compounds except **5** and **9** exhibited almost the same uptake enhancing effect. Similar comparative study within the butyl derivatives on the uptake of VLB into $KBCh^R$-8-5 cells revealed that the compounds follow the order: **18 > 19 >13 > 16 > 17 > 14 >11 > 12 > 15**. Further**,** the effects of varying concentrations of modulators **3-6, 12-14, 16, 18** or VRP on the uptake of VLB in MDR cells were studied. The $KBCh^R$-8-5 cells were exposed for 2 hours to 49.9 nM [^{3}H] VLB in the absence or presence of 5, 10, 25, or 50 μM concentration of the above nine compounds or VRP and the intracellular concentration of VLB in picomoles/10^6 cells was calculated. Examination of the data revealed that all the modulators at 5 μM exhibited the least VLB enhancing effect whereas at 10 μM, 25 μM, or 50 μM the modulators exhibited a significant increase on the accumulation of VLB suggesting that the uptake of VLB into $KBCh^R$-8-5 cells is dependent on the concentration of modulator. The greatest increase was for derivative **18** and this was probably due to the enhanced lipophilicity of the compounds after -Br substitution. Additionally, it is speculated that the acridone nucleus with -Br at position C-2 may exhibit a higher affinity for membranes or be more readily taken up into cells than that with a hydrogen atom. Further, it is interesting to note that the parent compound **1** was the least effective in increasing VLB uptake suggesting that N^{10}-substitution is required for optimal activity. Thus, the presence of an N^{10}-substitution seems to be necessary to optimize the activity in 2-bromoacridones. Comparison of the derivatives for their

Table 1. Effect of 2-Bromo-N^{10}-Substituted-Acridones on the Accumulation of VLB in KBChR-8-5 Cells



Comp. No.	R	pK_a	$log_{10}P^a$	Vinblastine uptake (% control)[b]
1	—H	8.35	1.97	652
2	$-CH_2-CH_2-CH_2-Cl$	8.90	1.64	1046
3	$-(CH_2)_3-$N(piperazine)$-CH_3$	5.27 9.22	1.40	981
4	$-(CH_2)_3-$N(piperidine)	5.42 9.40	1.49	909
5	$-(CH_2)_3-$N(morpholine)	4.85 9.25	1.62	1218
6	$-(CH_2)_3-$N(pyrrolidine)	5.27 9.36	1.42	989
7	$-(CH_2)_3-N(CH_2-CH_3)_2$	5.39 9.26	1.52	997
8	$-(CH_2)_3-N(CH_2-CH_2-OH)_2$	4.73 9.35	1.47	975
9	$-(CH_2)_3-$N(piperazine)$-CH_2-CH_2-OH$	5.32 9.30	1.55	1295
10	$-(CH_2)_3-$N(thiomorpholine)	4.90 9.40	1.61	920
11	$-CH_2-CH_2-CH_2-CH_2-Cl$	8.81	1.84	1245
12	$-(CH_2)_4-$N(piperazine)$-CH_3$	5.61 9.32	1.77	1232
13	$-(CH_2)_4-$N(piperidine)	5.30 9.51	2.03	1693
14	$-(CH_2)_4-$N(morpholine)	4.93 9.22	1.87	1275
15	$-(CH_2)_4-$N(pyrrolidine)	5.32 9.42	1.74	1157
16	$-(CH_2)_4-N(CH_2-CH_3)_2$	5.46 9.43	1.95	1401

(Table 1. Contd….)

Comp. No.	R	pK_a	$log_{10}P$[a]	Vinblastine uptake (% control)[b]
17	$-(CH_2)_4-N(CH_2-CH_2-OH)(CH_2-CH_2-OH)$	4.73 9.27	1.85	1326
18	$-(CH_2)_4-N$ (piperazine) $N-CH_2-CH_2-OH$	5.76 9.47	2.11	1916
19	$-(CH_2)_4-N$ (thiomorpholine) S	5.24 9.32	2.0	1825

[a]Octanol / water partition coefficient

[b] $\left(\frac{\text{Vinblastine uptake with modulator}}{\text{Vinblastine uptake without modulator}}\right)$ x100

Compounds were tested at their IC_{50} concentrations. Each experiment was done in triplicate with a SD of less than 8% of the mean.

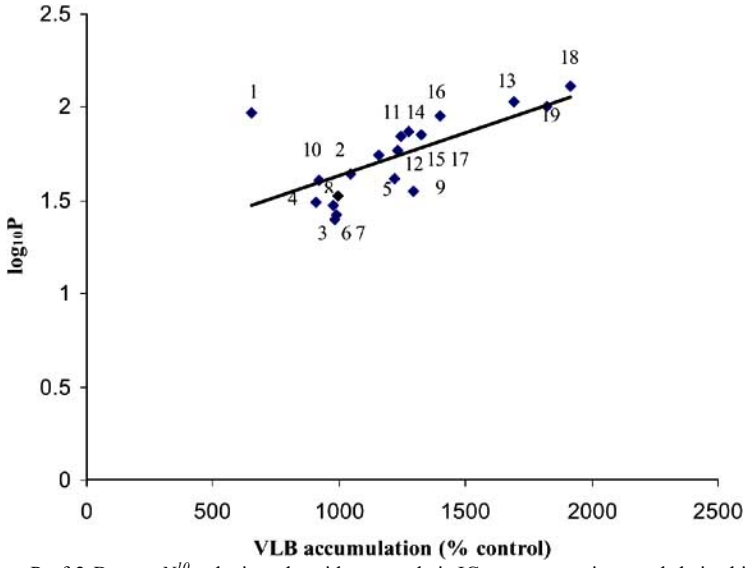


Fig.(**1**). Correlation between the $log_{10}P$ of 2-Bromo-N^{10}-substituted acridones at their IC_{50} concentrations and their ability to enhance VLB uptake in $KBCh^R$-8-5 cells ($R^2 = 0.50$).

ability to potentiate the uptake of VLB in $KBCh^R$-8-5 cells revealed that they follow the order N^{10}-butyl > N^{10}-propyl, although there are a few exceptions.

In the efflux assay, the cell associated radiolabel remaining after 2 hours was determined and calculated as a percentage of the VLB present after loading and the data are given in Table **2**. The results showed that VRP (100 μM) and each of the modulators at their IC_{50} values (Table **3**) significantly inhibited the efflux of VLB, suggesting that they may be competitors for P-gp. More than 80% of VLB was lost from the cells within 2 hours of incubation in the absence of modulator, whereas, 50-90% of VLB in the presence of modulators (**1-19**) or 68% of VLB in the presence of VRP was retained in the cells. From among the nineteen modulators, eleven compounds except (**1-2, 4, 5, 7, 8, 10, and 19**) exhibited greater efflux inhibiting activity than VRP. Thus, this data suggests that the 2-bromoacridones like VRP are able to inhibit P-gp mediated VLB efflux from MDR $KBCh^R$-8-5 cells.

To Investigate whether 2-Bromoacridones Interact with P-glycoprotein by Photolabeling this Protein with [^{3}H] Azidopine

It has been previously demonstrated that P-gp is specifically labeled with VLB analogues and that VRP blocked the specific labeling [41]. The VLB transport and cell survival data suggest that the modulatory effect of 2-bromoacridones is through an interaction with P-gp. To confirm this suggestion the authors have examined the competition between [^{3}H] azidopine and nineteen 2-bromoacridones. At their IC_{50} concentrations, 2-bromoacridones inhibited the labeling of P-gp by [^{3}H] azidopine. The binding of [^{3}H] azidopine to P-gp after inhibition by acridones

Table 2. Effect of 2-Bromoacridones on the Cellular Retention of VLB in KBCh^R-8-5 Cells After 2 Hours Efflux

Comp. No[a]	VLB retention (%)	Comp. No[a]	VLB retention (%)
1	50	11	65
2	56	12	85
3	80	13	83
4	67	14	78
5	75	15	72
6	70	16	85
7	60	17	85
8	69	18	90
9	83	19	75
10	58	Control	20
		VRP	68

All values represent the mean of two separate experiments with a S.D. of less than 6% of the mean; each experiment was done in triplicate.

[a]Modulators used at their IC_{50} concentrations.

expressed in percentage of control (no competitor) is as follows: **1** by 63%, **2** by 75%, **3** by 57%, **4** by 48%, **5** by 38%, **6** by 25%, **7** by 59%, **8** by 60%, **9** by 19%, **10** by 57%, **11** by 68%, **12** by 27%, **13** by 12%, **14** by 19%, **15** by 20%, **16** by 19%, **17** by 20%, **18** by 10%, **19** by 15% and VRP by 61%. Comparison of the data on the competition between [^{3}H] azidopine and each acridone modulator at its IC_{50} concentration revealed that the ability of all the compounds, except compounds **2,** and **11,** to inhibit the [^{3}H] azidopine labeling of P-gp, is greater than that of the standard modulator VRP. If a modulator inhibits labeling by the probe of interest, then it is said that this modulator probably functions by competing for the drug-binding site on the protein. The fact that all the compounds have reduced the photoaffinity labeling of azidopine appreciably, the results predict that the modulators compete for azidopine for binding to P-gp. Careful evaluation of the data of the modulators showed that butyl derivatives have exhibited greater competition than that of propyl derivatives suggesting that this is due to enhanced lipophilicity of the compounds. Comparison of the inhibition data has revealed that those modulators, which have exhibited greater VLB accumulation effect and maximum VLB efflux preventing ability from MDR cells, have exhibited greater competition for azidopine labeling, suggesting that the activity of 2-bromoacridones may be mediated through P-gp-dependent mechanism.

Table 3. Cytotoxicity of 2-Bromo-*N*^{10}-Substituted-Acridones in Human KBCh^R-8-5 Cells

Comp. No.	IC_{10} (μM)	IC_{50} (μM)	Comp. No.	IC_{10} (μM)	IC_{50} (μM)
1	0.61	19.00	11	1.45	17.00
2	0.80	16.50	12	1.72	9.00
3	1.50	14.00	13	0.68	6.65
4	1.10	14.00	14	0.78	9.20
5	1.70	20.00	15	0.60	18.10
6	0.84	13.50	16	0.61	8.20
7	1.70	14.00	17	0.62	5.65
8	2.70	12.50	18	0.40	3.70
9	1.40	8.50	19	0.52	8.40
10	1.00	17.00	--	--	--

IC_{10} and IC_{50} are the concentrations (μM) required to produce 10% and 50% reduction respectively, in clonogenic survival of MDR cells under the conditions described in the experimental section.

In Vitro Cytotoxicity of Acridones in Drug-Sensitive and Resistant Cancer Cell Lines

The cytotoxicity of nineteen acridones was examined by incubating $KBCh^R$ -8-5 cells continuously for 7 days with several concentrations of acridones. The concentration of modulators that reduced colony formation by 10% and 50% (IC_{10} and IC_{50}) were determined from concentration percent survival curves and the results are given in Table **3**. The IC_{10} and IC_{50} values for N^{10}-substituted acridones lie respectively in the range 0.40 to 2.70 μM and 3.70 to 20 μM for $KBCh^R$-8-5 cells. Examination of IC_{50} values for N^{10}-chloropropyl-substituted-2-bromoacridone and N^{10}-chlorobutyl-substituted-2-bromoacridone derivatives against $KBCh^R$-8-5 cells revealed that antiproliferative activity relatively increased as the chain length increased from 3 to 4 suggesting that the hydrophobicity plays an important role on the biological activity. The increase of distance between the ring nucleus and amino group increased the anti-proliferative activity of these compounds. It is clear from the data that the comparison of the cytotoxicity of the butyl derivatives has shown that the cell killing potency follows the order **18 > 17 > 13 > 15> 16 >19 > 12 > 14 >11**. Therefore, from this study we can tentatively conclude that the structural features required within the series to cause a maximum anti-proliferative activity in $KBCh^R$-8-5 cells include hydrophobic acridone ring nucleus with a side chain tertiary cationic amino group that is separated from the aromatic ring by at least three to four carbons. However, it is not possible to draw conclusions about the correlation between structure and antiproliferative activity from these studies.

Sensitization of Drug-Resistant $KBCh^R$-8-5 Cells by 2-Bromo-N^{10}-substituted acridones

We studied the ability of 19 compounds to modulate the cytotoxicity of VLB in drug-sensitive KB-3-1 and its MDR subline $KBCh^R$-8-5 cell line. Cells (KB-3-1, or $KBCh^R$-8-5) were exposed continuously to 0 - 100 nM VLB for 7-days in the absence or presence of IC_{10} concentrations of acridone modulators and the fold-potentiation of VLB cytotoxicity for $KBCh^R$-8-5 are summarized in Table **4**. The modulators enhanced the cytotoxicity of VLB by 3.8 to 34-fold against $KBCh^R$-8-5 cells. The IC_{50} values of VLB against $KBCh^R$-8-5 cells in the presence of IC_{10} of modulators (**1-19**) lie in the range of 2.1-18.1 nM. Examination of IC_{50} values of VLB in the presence of propyl derivatives (4.0-18.1 nM) or butyl derivatives (2.1-8.5 nM) of acridone has revealed that the butyl derivatives have sensitized the MDR $KBCh^R$-8-5 cells to a greater extent presumably due to increased hydrophobicity. Comparative study of the abilities of the modulator to potentiate the cytotoxicity of VLB in the presence of acridone modulators revealed that the modulators (**18, 15 and 13**) demonstrated the greatest effect followed by **16, 14, 12, 17** and so on. Only five acridone derivatives (**13 - 16 and 18**), like VRP, were able to completely reverse the 25-fold resistance of $KBCh^R$-8-5 cells to VLB. The IC_{50} values for continuous exposure to VLB was 3.0 nM in KB-3-1 and 69.0 nM in $KBCh^R$-8-5 cells in the absence of modulating agent. The most effective modulators (**13-16 and 18**) in $KBCh^R$-8-5 cells were subsequently tested in KB-3-1 and all were shown to cause a small sensitization (2.0-3.0-fold) of this drug-sensitive line to VLB. However, a similar degree of sensitization was also found when the classical MDR modulator, VRP (3.8-fold) was used

Thus, the structural features required for significant reversal of Pgp-mediated MDR include a tricyclic hydrophobic acridone ring with a –Br group at position C-2 and a bishydroxyethylamino, *N*-methylpiperazino, morpholino, (β-hydroxyethyl)piperazino, piperidino, pyrrolidino or thiomorpholino side chain containing a tertiary amino group at a distance of at least three to four carbon atoms from the

Table 4. Effect of 2-Bromoacridones on the Potentiation of VLB Cytotoxicity in Drug Resistant $KBCh^R$-8-5 Cells

Comp. No.[a]	VLB IC^b_{50} (nM)	Fold- potentiation[c]	Comp. No.[a]	VLB IC^b_{50} (nM)	Fold- potentiation[c]
1	18.10	3.80	11	8.50	8.00
2	13.50	5.03	12	3.00	22.66
3	9.10	7.50	13	2.10	Complete[d]
4	8.00	8.50	14	2.21	Complete
5	4.20	16.20	15	2.10	Complete
6	8.60	7.90	16	2.14	Complete
7	8.80	7.27	17	3.75	18.13
8	7.00	9.71	18	2.10	Complete
9	4.00	17.00	19	5.90	11.52
10	11.50	5.91	--	--	--

[a] Modulators used at the IC_{10} concentration (Table 3).

[b]Concentration of modulators required for 50% reduction of cells compared to controls.

[c]Fold-potentiation.

[d]Complete reversal of VLB resistance.

tricyclic ring. It is not known whether a butyl-bridge is the distance required for optimal activity because no compounds in this series having alkyl chain length longer than four carbons were studied.

It is apparent, however, that the compounds most effective at enhancing VLB accumulation (**13-16 and 18**) are among the compounds that can completely reverse VLB resistance in $KBCh^R$-8-5 cells. The structure-activity relationship suggests that for this class of drugs, compounds with a tertiary amino group incorporated into a tricyclic ring structure in a particular spatial orientation and at a distance of at least three to four carbons from the hydrophobic acridone ring are optimal for reversing MDR. These findings are in agreement with the previous studies of phenoxazine, phenothiazine and thioxanthene class of chemosensitizers, as well as those of others with derivatives of indole alkaloids. The same factors that determine the potency of 2-bromo-acridones may also determine the activity of other classes of compounds.

Correlation between Anti-calmodulin Activity and Inhibition of Cellular Growth and MDR

Previously it has been demonstrated that calmodulin inhibitors are capable of sensitizing MDR cells by possibly interfering with cellular drug accumulation [42, 43]. To check the role of calmodulin as a possible target for the effect of modulators on cellular growth and MDR, we have determined the IC_{50} values for the inhibition of calmodulin (Table **5**) by 2-bromoacridones and compared with their IC_{50} values for the inhibition of cellular growth and also with their effect on Pgp-mediated MDR. The evaluation of anti-calmodulin activity is based on the measurement of phosphodiesterase activity in the presence or absence of acridones. The method involves the hydrolysis of *c*AMP to AMP, a product of *c*AMP–phosphodiesterase reaction, followed by quantitation by HPLC method. Minimum detection level of AMP by this method was 0.05 μg and was linear up to 3.13 μg. In the absence of calmodulin, phosphodiesterase had a low basal activity of 0.064 μmol mg^{-1} min^{-1}. In order to find out the substrate saturation point, *c*AMP was varied from 0.15 to 91 nmol in the reaction mixture containing 1.25 x 10^{-4} U of phosphodiesterase. K_m of the enzyme for *c*AMP was found to be 4 x 10^{-5} M and V_{max} was 326.8 nmol of AMP hydrolyzed mg^{-1} min^{-1} of protein. The hydrolysis of *c*AMP by the enzyme was studied as a function of incubation time and the rate of formation of AMP was linear for at least 90 min, after which the activity decreased presumably due to the depletion of the substrate concentration in the reaction mixture. The pH of the working buffer was also varied between 3 and 11 and an optimum pH of 7.5 was found for maximum enzyme activity, which closely approximates that found by others [44, 45]. In order to determine the effect of calmodulin for maximum phosphodiesterase activity, varied concentrations of activator upto 0.2 μg (0.0 - 1.0 U) were incubated with 1.24 x 10^{-4} U of phosphodiesterase containing 0.043 μmol of *c*AMP and the activity determined as described in Experimental Section. At low concentration of the activator, the stimulation was small. As the concentration of the activator increased, the extent of stimulation was proportionately greater, reaching a plateau at about 0.12 μg (0.6 U) of calmodulin. Half-maximal activation was obtained at 6.6 x 10^{-3} μg (0.033 U) of activator. With saturating concentration of the activator, the enzyme activity was stimulated approximately by 5-fold. As activation of the enzyme was completely dependent on the addition of calmodulin and Ca^{2+}, a 2.3 μM concentration of calcium was found to be essential for 50% of maximal activation and further demonstrated that Mg^{2+} cannot substitute for Ca^{2+} in the activation of the enzyme. In the present HPLC method, AMP and *c*AMP were directly separated from the reaction mixture and quantitated to assay phosphodiesterase, calmodulin activation of phospho-diesterase and inhibition of calmodulin activation by acridones. This method is simple, rapid, reproducible and a microassay to measure the activity of phosphodiesterase. The method offers several advantages over commonly used procedures, the major one being its extreme sensitivity. The anti-calmodulin activity was determined by assaying the phosphodiesterase activity in the presence or absence of acridones (**1, 3, 4, 8, 9, 12, 13, 17, 18**) in the range of concentration 0.001–100 μM and IC_{50} values were determined from the dose–response curves. Table **5** shows the activity of various acridones towards the calmodulin-dependent enzyme activity. None of the compounds gave any significant inhibition of the calmodulin independent *c*AMP-phosphodiesterase activity. These results demonstrate that the acridone derivatives only inhibit the Ca^{2+}/calmodulin stimulated *c*AMP-phosphodiesterase activity and have no direct effects on the enzyme itself. The IC_{50} values lie in the range of 4–16 μM for the most potent modulators (**3, 4, 8, 9, 12, 13, 17**, and **18**). 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 **18** (IC_{50} = 4.0 μM), **17** and **13** (IC_{50} = 5.30 μM and 5.80 μM respectively), suggesting that the variation of

Table 5. Inhibition of Calmodulin-Dependent cAMP-Phosphodiesterase by 2-Bromoacridones

Compd. No.	Inhibition of PDE activation IC_{50} (μM)
1	>100.00
3	16.00
4	13.50
8	10.80
9	7.30
12	8.50
13	5.80
17	5.30
18	4.00

Antagonism of calmodulin by acridones was determined by their ability to inhibit by 50% the activation of a calmodulin-dependent form of cyclic nucleotide phosphodiesterase, as described in experimental section. IC_{50} values are the mean of three experiments with a S.D. of less than 5% of the mean.

the side chain is a strict requirement for activity. Most of the potent calmodulin inhibitors [42, 46-50] reported so far contain large hydrophobic region and carry positive charge at neutral pH. Both the hydrophobic region and charged amino group appear to be important for inhibiting calmodulin activity. Assessment of the structural requirements of the previously reported potent inhibitors revealed that the anti-calmodulin compounds contain the following: large hydrophobic trycyclic ring systems; an alkyl side chain containing at least three to four carbon atoms; and a tertiary amine group at the terminal end of the alkyl bridge. 2-bromoacridones fulfill these requirements and it is possibly this combination of structural features that causes the acridone derivatives to be effective in inhibiting the activity of calmodulin. Further, the relationships between anti-calmodulin activity and cytotoxicities in $KBCh^R$-8-5 cells for acridone derivatives were studied and the results showed a strong positive correlation between the inhibition of calmodulin-dependent *c*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. These results suggest that inhibition of calmodulin, an intracellular calcium-binding protein that is known to play a key role in regulating cell proliferation [51], may be of importance in mediating the cytotoxic effects of acridones.

CONCLUSION

We have successfully employed simple and improved synthetic routes for the preparation of acridones, which have proven to be one of a number of diverse agents capable of reversing Pgp-mediated MDR. Five acridones were able to completely reverse the resistance of the cells to VLB. At present, it is premature to make conclusions concerning the achievable plasma and tumor levels of acridones in animals or ultimately patients. Based on these data, acridones may also confer clinical benefits in the MDR setting. In summary, our results indicate that combining a good calmodulin inhibitor with an anti-cancer compound might provide a good strategy that could ultimately yield new modality for the treatment of refractory tumors.

EXPERIMENTAL SECTION

Reactions were monitored by TLC. Column Chromatography utilized silica gel Merck Grade 60 (230-400 mesh, 60 A^0). Melting points were recorded on a Tempirol hot-stage with microscope and are uncorrected. UV spectra were recorded in MeOH on a Shimadzu-UV-1601 spectrophotometer; IR spectra were recorded on a Perkin-Elmer Model 1320 spectrophotometer as KBr pellets. Elemental analyses were performed at Central Drug Research Institute, Lucknow, India. Found values are within 0.4% of theoretical values unless otherwise noted. ^{1}H- and ^{13}C-NMR spectra were recorded in DMSO-d_6 solution in a 5-mm tube on a Bruker drx 500 Fourier tranform spectrometer with tetramethylsilane as internal standard. The spectrometer was internally locked to deuterium frequency of the solvent. To obtain molecular weight information, acridone derivatives were analyzed by electro spray ionization (ESI) and electron ionization (EI) mass spectrometry.

All the chemicals and supplies were obtained from standard commercial sources unless otherwise indicated. DMEM media, Hank's balanced salt and trypsin–EDTA were purchased from Imperial (UK). Vinblastine sulfate was purchased from Cetus Corporation (Emery-ville, CA, USA). RPMI-1640 medium with glutamine and without sodium bicarbonate and sodium pyruvate were purchased from Gibco BRL (Grand Island, NY, USA). [^{3}H]-vinblastine (sp. Act. 9.4 Ci mmol^{-1})was purchased from Amersham Pharmacia Biotech, UK, Ltd. Verapamil hydrochloride, colchicine, dimethyl- sufoxide, *c*AMP, AMP, phosphodiesterase 3',5'- cyclic nucleotide and phosphodiesterase 3',5'-cyclic nucleotide-activator (calmodulin), DEAE cellulose and Sephadex G-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of 4′-Bromodiphenylamine–2 Carboxylic Acid

Ullmann Condensation

To a mixture of *o*-chlorobenzoic acid (10 g, 0.064 mol), *p*-bromoaniline (11g, 0.064 mol) and copper powder (0.2 g) in 60 mL isoamylalcohol, dry potassium carbonate (10 g) was slowly added and the contents were refluxed for 6 hours on an oil bath. The isoamylalcohol was removed by steam distillation and the mixture poured into one litre of hot water and acidified with concentrated hydrochloric acid. Precipitate formed was filtered, washed with hot water and collected. The crude acid was dissolved in aqueous sodium hydroxide solution, boiled in the presence of activated charcoal and filtered. On acidification of the filtrate with concentrated hydrochloric acid, light yellowish precipitate was obtained which was washed with hot water and recrystallized from aqueous methanol to give a light yellow solid (yield 14g, 72%, mp 160 ^{o}C). MS (m/z) 292 ($M^{+\cdot}$, 50).

2-Bromoacridone (**1**)

Nine grams of 4′-Bromodiphenylamine–2 carboxylic acid was taken in a flask to which was added 90 g of polyphosphoric acid. Shaken well and heated on a water bath at 100^{o}C for 3 hours. Appearance of yellow color indicated the completion of the reaction. Then, it was poured into 1 liter of hot water and made alkaline by liquor ammonia. The yellow precipitate that formed was filtered, washed with hot water and collected. The sample of 2-bromoacridone (**1**) was recrystallized from acetic acid (yield 10g, 90%, mp 385^{o}C). UV λ_{max} (ε) 215(6500), 258(9,932), 386(1768), 407(494) nm; IR 3436, 2987, 2855, 1636, 1526, 1466, 1277, 1036, 870, 676, 562 cm^{-1}; 1H-NMR δ 7.18-8.21 (m, Ar-H, 7H, H1, H3, H4 and H5-H8) and 12.06 (s,1H, NH); 13C-NMR δ 127.92 (C1), 120.38 (C_2),126.00 (C_3),120.09 (C_4), 117.63 (C_5), 135.95 (C_6), 121.51(C_7), 133.82 (C_8), 175.64 (C_9), 113.08 ($C_{1\cdot}$), 139.77 ($C_{4\cdot}$), 140.79 ($C_{5\cdot}$) and 121.75 ($C_{8\cdot}$); MS m/z (%) 274 [$M^{+\cdot}$, 50]. Anal. ($C_{13}H_8NOBr$) C, H, N.

10-(3'-*N*-Chloropropyl)-2-bromoacridone (**2**)

To the solution of 5 g (0.02 mol) of **1** dissolved in 20 mL tetrahydrofuran, was added 80 mL of 6N potassium hydroxide and 2 g (0.006 mol) of tetrabutylammonium bromide. The reaction mixture was stirred at room temperature for half an hour and added 1-bromo-3-chloropropane (0.065 mol) slowly into the reaction mixture stirred for 24 hours at room

temperature. Tetrahydrofuran was evaporated and the aqueous layer extracted with chloroform. The chloroform layer was washed with water and organic layer dried over anhydrous sodium sulfate and rotavaporated. The crude product was purified by column chromatograph to give a yellow solid of 10-(3'-chloropropyl)-2-bromoacridone (**2**) (yield 6g, 51%, mp 122°C). UV λ_{max} (ε) 218(10,617), 255 (19,775), 391(3704), 410(2625) nm; IR 3384, 2896, 1636, 1610, 1489, 1259, 803, 752, 576 cm^{-1}; ^{1}H-NMR δ 7.36-8.38 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5-H_8), 3.85-3.91(t, 2H, H_m), 3.79-3.81 (t, 2H, H_k) and 2.32-2.50 (m, 2H, H_l); ^{13}C-NMR δ 128.97 (C_1), 121.52 (C_2), 126.91 (C_3), 114.62 (C_4), 113.80 (C_5), 136.19 (C_6), 121.31(C_7), 134.09 (C_8), 175.76 (C_9), 122.85 ($C_{1'}$), 139.99 ($C_{4'}$), 141.01($C_{5'}$), 116.97 ($C_{8'}$), 41.85 (C_k), 30.19 (C_l) and 44.08 (C_m); MS m/z (%) 351[$M^{+.}$, 51]. Anal. ($C_{16}H_{13}NOClBr$) C, H, N.

10-[3'-*N*-(Methylpiperazino)propyl-2-bromoacridone (3)

To the solution of 1 g (2.9 mmol) of **2** dissolved in 60 mL of anhydrous acetonitrile, 1.31g of KI and 2.18 g of K_2CO_3 were added and refluxed for 30 minutes. Then added 1.1 g (11 mmol, 1.22 mL) of 1-methylpiperazine into it slowly and refluxed for 15 hours until a substantial amount of the product was formed as evidenced by TLC. The contents were cooled, diluted with water and extracted with chloroform. The chloroform layer was washed with water thrice, dried over anhydrous sodium sulfate and evaporated to give an oily product. The oily residue was purified by column chromatography to give a light yellow oil of 10-[3'-N-(methylpiperazino)propyl]-2-bromoacridone (**3**). An acetone solution of the free base was treated with ethereal hydrochloride to give the hydrochloride salt that was dried under high vacuum to get pure solid (**3**) (yield 1.2 g, 58%, mp 278-283°C). UV λ_{max} (ε) 217(7229), 258(9,770), 392 (2038), 407(1857) nm; IR 3406, 2863, 1635, 1460, 1268, 937, 768, 674, 570 cm^{-1}; ^{1}H-NMR δ 7.26-8.34 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5-H_8), 2.80-3.77 (m, 12H, H_a, H_b, H_c, H_d, H_k and H_m), 2.25 (s, 3H, H_e) and 2.27-2.43 (m, 2H, H_l); ^{13}C -NMR δ 128.68 (C_1), 121.29 (C_2), 126.57 (C_3), 114.19(C_4), 113.58 (C_5), 136.31(C_6), 121.30 (C_7), 134.93 (C_8),175.38 (C_9),122.36 ($C_{1'}$), 139.34 ($C_{4'}$), 141.36 ($C_{5'}$), 116.39 ($C_{8'}$), 44.16 (C_k), 23.09 (C_l), 42.49 (C_m), 50.09 (C_a and C_b), 51.45 (C_c and C_d) and 27.58 (C_e); MS m/z (%) 414[$M^{+.}$, 50].

10-(3'-*N*-Piperidinopropyl)-2-bromoacridone (4)

The procedure used for **3** was repeated with 1.19 g (3.40 mmol) of **2**, 1.56 g of KI, 2.6 g of K_2CO_3 and 1.2 mL (14.1 mmol) of piperidine. The product was purified by column chromatography to give a yellow oily product which was converted into hydrochloride salt **4** (yield 0.92 g, 47%, mp 130 °C). UV λ_{max} (ε) 216 (22,815), 255 (36,774), 389 (6928), 406 (5404) nm; IR 3443, 2936, 1625, 1450, 1294, 947, 767, 547 cm^{-1}; ^{1}H-NMR δ 7.37-8.41(m,7H, Ar-H, H_1, H_3, H_4 and H_5-H_8), 3.31-3.41 (m, 8H, H_k, H_m, H_a and H_b), 2.25-2.89 (m, 6H, H_l ,H_c and H_a) and 1.69-1.84 (m, 2H, H_e); ^{13}C-NMR δ 126.75 (C_1), 122.35 (C_2), 125.31(C_3), 118.68 (C_4), 116.04 (C_5), 134.81(C_6), 121.94 (C_7), 133.99 (C_8), 175.61(C_9), 126.06($C_{1'}$), 139.90($C_{4'}$), 141.15($C_{5'}$), 121.35 ($C_{8'}$), 52.90 (C_k), 21.39 (C_l), 42.98 (C_m), 52.65 (C_a and C_b), 22.37 (C_c and C_d) and 21.21(C_e); MS m/z (%) 399[$M^{+.}$,50]. Anal. ($C_{21}H_{23}N_2OBr$) C, H, N.

10-(3'-*N*-Morpholinopropyl)-2-bromoacridone (5)

Repeated the procedure used for **3** with 1 g (3.0 mmol) of **2,** 1.38 g of KI, 2.3 g of K_2CO_3 and 1.12g (1.04 mL, 12.06 mmol) of morpholine. The oily product was purified by column chromatography. The light yellow oily product was dissolved in anhydrous acetone and treated with ethereal hydrochloride. Hydrochloride salt of 10-(3'-N-morpholino-propyl)-2-bromoacridone **5** was obtained (yield 0.76 g, 40%, mp not determined due to hygroscopic nature). UV λ_{max} (ε) 215(18,178), 255(10,827), 389(3208), 407(1871) nm; IR 3422, 2954, 2816, 1641, 1262, 987, 916, 859, 614 cm^{-1}; ^{1}H-NMR δ 7.36-8.41 (m, 7H, Ar-H, H_1, H_3, H_4 and H_5-H_8), 3.04-3.10 (t, 4H, H_c and H_d), 2.50 (t, 4H, H_a and H_b), 2.27 (m, 4H, H_k and H_m), 2.27 (m, 4H, H_k and H_m,) and 1.21-1.97 (t, 2H, H_l); ^{13}C-NMR δ 126.77 (C_1), 122.34 (C_2), 125.33 (C_3), 118.15 (C_4), 115.64 (C_5), 135.32 (C_6), 121.99 (C_7), 134.47 (C_8), 175.34 (C_9), 126.52 ($C_{1'}$), 139.72 ($C_{4'}$), 141.00 ($C_{5'}$), 121.08 ($C_{8'}$), 53.08 (C_k), 21.12 (C_l), 42.57(C_m), 51.39 (C_a and C_b) and 63.27 (C_c and C_d); MS m/z (%) 401[$M^{+.}$,50]. Anal. ($C_{20}H_{21}N_2O_2Br$) C, H, N.

10-(3'-*N*-Pyrrolidinopropyl)-2-bromoacridone (6)

The procedure used for **3** was repeated with 1 g (3.0 mmol) of **2**, 1.38 g of KI, 2.3 g of K_2CO_3 and 1g (1.2 mL, 14.08 mmol) of pyrrolidine. The light yellow oily product was converted into hydrochloride salt of 10-(3'-N-pyrrolidinopropyl)-2-bromoacridone **6** (yield 0.76 g, 40%, mp 240-242°C). UV λ_{max} (ε) 218(18,596), 256(19,237), 389(3619), 407(1925) nm; IR 3384, 2956, 2810, 1646, 1267, 962, 752, 667, 553 cm^{-1}; ^{1}H-NMR δ 7.38 - 8.34 (m, Ar-H, 7H, , H_l, H_3, H_4 and H_5-, H_8), 3.14-3.94 (m, 8H, H_k, H_m, H_a and H_b) and 1.56-2.50 (m, 6H, H_l, H_c and H_d); ^{13}C-NMR δ 128.60 (C_1), 122.83 (C_2), 126.87 (C_3), 120.12 (C_4), 118.73 (C_5), 136.74 (C_6), 122.12 (C_7), 134.97 (C_8), 175.34 (C_9), 127.88 ($C_{1'}$), 140.24 ($C_{4'}$), 141.18 ($C_{5'}$), 121.46 ($C_{8'}$), 57.53 (C_k), 23.46 (C_l), 51.26 (C_m), 53.33 (C_a and C_b) and 22.90 (C_c and C_d); MS m/z (%) 385[$M^{+.}$, 50]. Anal. ($C_{20}H_{21}N_2OBr$) C, H, N.

10-(3'-[*N*-Diethylamino]propyl)-2-bromoacridone (7)

Amounts of 1.11 g (3.20 mmol) of **2,** 1.45 g of KI, 2.42 g K_2CO_3 and 1.2 mL (16.02 mmol) of diethylamine were refluxed and processed according to the procedure used for **3**. The crude product was purified by column chromatography and converted into its hydrochloride salt of 10-(3'-N-diethylamino) propyl)-2-bromoacridone **7** (yield 0.57 g, 46%, mp 210-214°C). UV λ_{max} (ε) 217(15,480), 256(31261), 392(5762), 411(4128) nm; IR 3396, 2985, 1629, 1489, 1459, 1260, 806, 755, 674, 556 cm^{-1}; ^{1}H-NMR δ7.28-8.34 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5-H_8), 3.10-3.45 (m, 8H, H_k, H_m, H_a and H_b), 2.14-2.50 (m, 2H, H_l) and 1.22-1.25 (m, 6H, H_c and H_d); ^{13}C-NMR δ 128.61 (C_1), 121.89 (C_2), 126.83 (C_3), 116.14 (C_4), 113.83 (C_5), 136.50 (C_6), 121.58 (C_7), 134.69 (C_8), 175.36 (C_9), 122.96 ($C_{1'}$), 140.32 ($C_{4'}$), 141.25 ($C_{5'}$), 118.94 ($C_{8'}$), 47.37 (C_k), 21.09 (C_l), 40.15 (C_m), 46.23 (C_a and C_b) and 8.53 (C_c and C_d); MS m/z (%) 388[$M^{+.}$,51]. Anal. ($C_{20}H_{23}N_2OBr$) C, H, N.

10-(3'-[*N*-[Bis(hydroxyethyl)amino]propyl]-2-bromoacridone (8)

The experimental procedure used for **3** is applicable with 1.16 g (3.30 mmol) of **2,** 1.52 g of KI, 2.53 g of K_2CO_3 and

1.28 g (1.20 mL, 9.83 mmol) of diethanolamine. The crude product was purified by column chromatography to give a light yellow solid of **8** (yield 0.8 g, 56%, mp 262-266°C). UV λ_{max} ( ) 217(11,077), 256(17,279), 392(3180), 411(2085) nm; IR 3395, 2971, 1634, 1489, 1284, 876, 754, 677 cm^{-1}; ^{1}H-NMR δ 7.32-8.38 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5-H_8), 4.51 (s, 2H, disappears on D_2O exchange H_e and H_f), 3.10-3.50 (m, 4H, H_c and H_d), 2.50-2.70 (t, 4H, H_k and H_m), and 1.30-1.80 (m, 2H, H_l); ^{13}C-NMR δ 128.38 (C_1), 122.55 (C_2), 126.65 (C_3), 116.04 (C_4), 113.74 (C_5), 136.11(C_6), 121.23 (C_7), 134.82 (C_8), 175.75 (C_9), 12188 ($C_{1'}$), 140.25 ($C_{4'}$), 141.22 ($C_{5'}$), 118.80 ($C_{8'}$), 51.61 (C_k), 19.03 (C_l), 43.95 (C_m), 56.27 (C_a and C_b) and 58.87(C_c and C_d); MS m/z (%) 419[$M^{+\cdot}$,51]. Anal. ($C_{20}H_{23}N_2O_3Br$) C, H, N.

10-(3'-[*N*-[(β-Hydroxyethyl) piperazino] propyl]-2-bromo-acridone (9)

The method employed for **3**was used with 1.25 g (3.70 mmol) of **2**, 1.64 g of KI, 2.73 g of K_2CO_3 and 1.52 g of (13.7mmol, 1.37 mL) of (β-hydroxyethyl)piperazine. The purified product was converted into its hydrochloride salt of **9** (yield 1.1 g, 51%, mp 140-142 °C). UV λ_{max} (ε) 218 (7709), 258 (13,845), 392 (2583), 410 (727) nm; IR 3356, 2938, 1643, 1420, 1289, 754, 648, 557 cm^{-1}; 1H-NMR δ 7.37-8.40 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5-H_8), 4.55 (s 1H, H_g), 3.18-3.80 (m, 4H, H_k, and H_l), 2.03-2.55 (m, 12H, H_a, H_b, H_c, H_d , H_e and H_m) and 1.00-1.90 (m, 2H, H_l); ^{13}C-NMR δ 128.57 (C_1), 122.79 (C_2), 126.82 (C_3), 115.98 (C_4), 113.95 (C_5), 136.73 (C_6), 121.41(C_7), 134.97 (C_8), 175.55 (C_9), 121.76 ($C_{1'}$), 140.22 ($C_{4'}$), 141.26 ($C_{5'}$), 118.75 ($C_{8'}$), 52.47 (C_k), 21.57 (C_l), 42.70 (C_m), 47.88 (C_a and C_b), 48.30 (C_c and C_d), 54.84 (C_e) and 57.37 (C_f); MS m/z (%) 445[$M^{+\cdot}$,50]. Anal. ($C_{22}H_{26}N_3O_2Br$) C, H, N.

10-(3'-*N*-Thiomorpholinopropyl)-2-bromoacridone (10)

Amounts of 1.13 g (3.20 mmol) of **2,** 1.59 g of KI, 2.58 g of K_2CO_3 and 1.52 g (12.6 mmol, 1.3 mL) of thiomorpholine were refluxed and processed according to the procedure used for **3**. The oily product was purified by chromatography and converted to hydrochloride salt of **10** (yield 1.1 g, 51%, mp 156-160 °C). UV λ_{max} (ε) 217(4289), 256(9,528), 392(926), 411(229) nm; IR 3331, 2781, 1606, 1457, 1289, 758, 675, 557 cm^{-1}; ^{1}H-NMR δ 7.24-8.39 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5-H_8), 4.32-4.45 (m, 3H, H_n), 3.50 (m, 2H, H_k), 1.75-2.50 (m, 2H, H_l) and 1.02-1.61 (m, 2H, H_m); ^{13}C-NMR δ 128.99 (C_1), 122.62 (C_2), 125.08 (C_3), 116.49 (C_4), 112.35 (C_5), 136.23 (C_6), 121.11 (C_7), 134.58 (C_8), 175.32 (C_9), 122.01 ($C_{1'}$), 140.28 ($C_{4'}$), 141.56 ($C_{5'}$), 118.71 ($C_{8'}$), 54.20 (C_k), 22.55 (C_l), 44.08 (C_m), 53.21 (C_a and C_b) and 26.84 (C_c and C_d); MS m/z (%) 417[$M^{+\cdot}$,50].

10-(4'-*N*-Chlorobutyl)-2-bromoacridone (11)

Compound **11** (yield 6.5 g, 60%) in the pure form was prepared by following the procedure used for **2** with 6 g (0.026 mol) of **1** and 1-bromo-4-chlorobutane (0.065 mmol). UV λ_{max} (ε) 218 (8,986), 258 (19,702), 391(2707), 410(1593) nm; IR 3416, 2871, 1634, 1485, 1257, 962, 784, 582 cm^{-1}; ^{1}H-NMR δ 7.20-8.40 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5-H_8), 4.45 (t, 3H, H_n), 3.14-3.45 (m, 2H, H_k), 1.47-1.76 (m, 2H, H_l) and 1.10-1.38 (m,2H, H_m); ^{13}C-NMR δ 126.78 (C_1), 122.51 (C_2), 125.40 (C_3), 118.47 (C_4), 115.95 (C_5), 134.48 (C_6), 121.64 (C_7), 133.75 (C_8), 175.37 (C_9), 125.86 ($C_{1'}$), 140.03 ($C_{4'}$), 141.29 ($C_{5'}$), 117.67 ($C_{8'}$), 44.70 (C_k), 25.51 (C_l), 24.26 (C_m) and 44.96 (C_n); MS m/z (%) 365[$M^{+\cdot}$,50]. Anal. ($C_{17}H_{15}NOClBr$) C, H, N.

10-(4'-*N*-(Methylpiperazino)butyl)-2-bromoacridone (12)

The procedure used for **3** was followed with 1.09 g (2.99 mmol) of **11**, 1.43 g of KI, 2.38 g of K_2CO_3 and 1.56 g (15 mmol) of N-methylpiperazine. Oily residue was then converted into hydrochloride salt (yield 1.20g, 55%, mp 151°C). UV λ_{max} (ε) 217 (13,011), 257 (20,487), 392 (3938), 411(2767) nm; IR 3416, 2928, 1609, 1457, 1276, 847, 752, 559 cm^{-1}; ^{1}H-NMR δ 7.22 -8.40 (m, Ar-H, 7H, H_1 and H_3-H_8), 4.05 (s, 3H, H_e), 3.23-3.69 (m, 12H, H, H_n, H_a, H_b, H_c and H_d), 1.84-2.83 (m, 4H, H_l and H_m); ^{13}C- NMR δ 126.77 (C_1), 122.51 (C_2), 125.37 (C_3), 118.73 (C_4), 116.12 (C_5), 134.61 (C_6), 121.76 (C_7), 133.84 (C_8), 175.44 (C_9), 125.90 ($C_{1'}$), 140.06 ($C_{4'}$), 141.31($C_{5'}$), 121.51 ($C_{8'}$), 55.20 (C_k), 20.23 (C_l), 20.06 (C_m), 45.16 (C_n), 49.43 (C_a and C_b), 48.05 (C_c andC_d) and 42.02 (C_e); MS m/z (%) 428[$M^{+\cdot}$,51].

10-(4'-*N*-Piperidinobutyl)-2-bromoacridone (13)

The procedure used for **4** was repeated with 1.08 g (3.09 mmol) of **11,** 1.41 g of KI, 2.36 g of K_2CO_3 and 1.25 g (14.7 mmol, 1.45 mL) of piperidine. The oily product was converted to hydrochloride salt of **13** (yield 1.02 g, 55%, mp 266-270°C). UV λ_{max} (ε) 217 (30,412), 254 (37,958), 390 (7472), 408 (5895) nm; IR 3509, 2983, 2701, 1633, 1279, 839, 752, 573 cm^{-1}; ^{1}H-NMR δ 7.37-8.41 (m, Ar-H, 7H, H_1 and H_3-H_8), 4.50 (t, 2H, H_k), 3.00-3.50 (m, 2H, H_n), 2.50-2.80 (m, 4H, H_a and H_b) and 1.00-1.90 (m, 10H, H_l, H_m, H_c, H_d, and H_e); ^{13}C-NMR δ 126.84 (C_1), 122.57 (C_2), 125.44 (C_3), 118.72 (C_4), 116.12 (C_5), 134.62 (C_6), 121.80 (C_7), 133.86 (C_8), 175.41 (C_9), 125.95 ($C_{1'}$), 140.14 ($C_{4'}$), 141.37 ($C_{5'}$), 121.58 ($C_{8'}$), 55.44 (C_k), 20.35 (C_l), 24.32 (C_m), 45.04 (C_n), 49.43 (C_a and C_b) and 48.05 (C_c and C_d); MS m/z (%) 413 [$M^{+\cdot}$, 50]. Anal. ($C_{22}H_{25}N_2OBr$) C, H, N.

10-(4'-*N*-Morpholinobutyl)-2-bromoacridone (14)

The experimental method used for **5** is applicable with 1.19 g (3.40 mmol) of **11**, 1.55 g of KI, 2.59 g of K_2CO_3 and 1.12 g (1.05 mL, 12.14 mmol) of morpholine. The oily product, which was purified by column chromatography, was converted into hydrochloride salt of **14** (yield 0.88 g, 43%, mp 158-160°C). UV λ_{max} (ε) 217(17,904), 256(28,309), 392(5069), 412(3972) nm; IR 3406, 2861, 1645, 1480, 1257, 839, 752, 573 cm^{-1}; ^{1}H-NMR δ 7.37-8.44 (m, Ar-H, 7H, H_1 and H_3-H_8), 4.50 (t,2H, H_k), 3.37-4.20 (m, 4H, H_c and H_d), 3.00-3.20 (m, 4H, H_a and H_b) and 1.00-2.00 (m, 4H, H_l and H_m); ^{13}C- NMR δ 128.49 (C_1), 122.65 (C_2), 121.99 (C_3), 115.99 (C_4), 113.83 (C_5), 136.72 (C_6), 121.31(C_7), 134.93 (C_8), 175.68 (C_9), 125.90 ($C_{1'}$), 140.06 ($C_{4'}$), 141.31($C_{5'}$), 121.51 ($C_{8'}$), 55.80 (C_k), 23.84 (C_l), 20.04 (C_m), 44.97 (C_n), 51.17 (C_a and C_b) and 63.21 (C_c and C_d); MS m/z (%) 415[$M^{+\cdot}$,50]. Anal. ($C_{21}H_{23}N_2O_2Br$) C, H, N.

10-(4'-*N*-Pyrrolidinobutyl)-2-bromoacridone (15)

Amounts of 1 g (2.74 mmol) of **11**, 1.31 g of KI, 2.18 g of K_2CO_3 and 0.77 mL (8.1 mmol) of pyrrolidine were refluxed and processed according to the procedure used for **6**. The yellow oily product was converted into hydrochloride

salt (yield 1.1 g, 77%, mp 85°C). UV λ_{max} (ε) 218 (16,293), 255 (29,759), 391(5167), 411(4190) nm; IR 3421, 2951, 1636, 1470, 1289, 849, 760, 579 cm^{-1}; ^{1}H-NMR δ 7.27-8.40 (m, Ar-H, 7H, H_l and H_3-H_8), 4.20-4.50 (m, 2H, H_k), 3.10-3.50 (m, 2H, H_n), 2.10-2.90 (m, 4H H_a and H_b), 1.83-1.98 (m, 4H, H_c and H_d) and 1.00-1.33 (m, 4H, H_l and H_m); ^{13}C-NMR δ 126.77 (C_1), 122.50 (C_2), 125.36 (C_3), 118.79 (C_4), 116.16 (C_5), 134.65 (C_6), 121.78 (C_7), 133.88 (C_8), 175.46(C_9), 125.90 ($C_{1'}$), 140.07 ($C_{4'}$), 141.32 ($C_{5'}$), 121.51 ($C_{8'}$), 53.61 (C_k), 17.56 (C_l), 24.20 (C_m), 45.15 (C_n), 50.41(C_a and C_b) and 22.84.(C_c and C_d); MS m/z (%) 400[$M^{+\cdot}$,51]. Anal. ($C_{21}H_{23}N_2OBr$) C, H, N.

10-(4'-[*N*-Diethylamino)butyl)-2-bromoacridone (16)

Repeated the procedure used for **7** with 1.2 g (3.30 mmol) of **11**, 1.57 g of KI, 2.62 g of K_2CO_3 and 1.3 mL (17.8 mmol) of diethylamine. The product was purified by column chromatography and converted to hydrochloride (yield 1.18 g, 54%, mp 173°C). UV λ_{max} (ε) 217(16441), 257(30326), 392(5840), 407(16,441) nm; IR 3405, 2944, 1625, 1284, 1030, 849, 756, 573 cm^{-1}; ^{1}H-NMR δ 7.27-8.40 (m, Ar-H, 7H, H_l and H_3-H_8), 4.40-4.80 (m,2H, H_k), 3.00-3.50 (m, 6H, H_a, H_b and H_n), 2.49-2.51 (t, 4H, H_a and H_b), 1.56-2.49 (m, 4H, H_l and H_m) and 1.22-1.31 (m, 6H, H_c and H_d); ^{13}C-NMR δ 126.80 (C_1), 122.57 (C_2), 125.41(C_3), 118.76 (C_4), 116.16 (C_5), 134.60 (C_6), 121.78 (C_7), 133.83 (C_8), 175.46 (C_9), 125.93 ($C_{1'}$), 140.13 ($C_{4'}$), 141.37 ($C_{5'}$), 121.55 ($C_{8'}$), 54.76 (C_k), 17.56 (C_l), 24.20 (C_m), 45.15 (C_n), 50.41(C_a and C_b) and 8.50 (C_c and C_d); MS m/z (%) 402[$M^{+\cdot}$,48]. Anal. ($C_{21}H_{25}N_2OBr$) C, H, N.

10-(4'-[*N*-[Bis-(hydroxyethyl)amino]butyl]-2-bromoacridone (17)

The method employed for **8** was used with 1.2 g (4.2 mmol) of **11**, 1.8 g of KI, 2.8 g of K_2CO_3 and 1.20 mL (9.83 mmol) of bis-hydroxyethylamine. The product was purified by column chromatography and converted to hydrochloride salt of **17** (yield 1.14 g, 54%, mp 250-255 °C). UV λ_{max} (ε) 217(9940), 257(9,940), 392(620), 407(124) nm; IR 3415, 2883, 1629, 1473, 1278, 1081, 808, 756, 569 cm^{-1}; ^{1}H-NMR δ 7.24-8.37 (m, Ar-H, 7H, H_l and H_3-H_8), 4.40 (s, 2H, H_e and H_f disappearing on D_2O exchange), 3.10-3.40 (m, 6H, H_c, H_n and H_k), 2.50 (s, 4H, H_a and H_b) and 1.00-2.00 (m, 4H, H_l and H_m); ^{13}C-NMR δ 126.74 (C_1), 122.49 (C_2), 125.33 (C_3), 118.79 (C_4), 116.17 (C_5), 134.55 (C_6), 121.65 (C_7), 133.82 (C_8), 175.47 (C_9), 125.82 ($C_{1'}$), 140.13 ($C_{4'}$), 141.39 ($C_{5'}$), 121.55 ($C_{8'}$), 54.13 (C_k), 23.67 (C_l), 24.24 (C_m), 45.60 (C_n), 56.55 (C_a and C_b) and 59.33 (C_c and C_d); MS m/z (%) 433[$M^{+\cdot}$,50]. Anal. ($C_{21}H_{25}N_2O_3Br$) C, H, N.

10-(4'-[*N*-[(β-Hydroxyethyl)piperazino]butyl]-2-bromoacridone (18)

The experimental method used for **9** was repeated with 1.2 g (3.30 mmol) of **11**, 1.57 g of KI, 2.62 g of K_2CO_3 and 2.1 mL (16.9 mmol) of β-hydroxyethyl piperazine. The product was purified by column chromatography and converted to hydrochloride salt by treating the residue with ethereal hydrochloride (yield 1.18 g, 54%, mp 144-147°C). UV λ_{max} (ε) 217(16,212), 256(38,085), 391(16540), 410 (14330) nm; IR 3406, 2937, 1642, 1458, 1261, 1067, 863, 757, 548 cm^{-1}; ^{1}H-NMR δ 7.28-8.41 (m, Ar-H, 7H, H_l and H_3-H_8), 4.50 (t, 1H, H_g), 3.06-3.80 (m, 4H, H_k and H_f), 2.08-2.50 (m, 12H, H_a, H_b, H_c, H_d, H_e and H_n) and 1.22-1.37 (m, 4H, H_l and H_m); ^{13}C-NMR δ 126.74 (C_1), 122.47 (C_2), 124.65 (C_3), 118.71(C_4), 116.11(C_5), 134.60 (C_6), 121.48 (C_7), 133.83 (C_8), 175.43 (C_9), 125.89 ($C_{1'}$), 140.03 ($C_{4'}$), 141.28 ($C_{5'}$), 121.48 ($C_{8'}$), 56.37 (C_k), 20.25 (C_l), 24.06 (C_m), 45.17 (C_n), 47.91 (C_a and C_b), 45.71(C_c and C_d), 49.38 (C_e) and 55.33(C_f); MS m/z (%) 458[$M^{+\cdot}$,51]. Anal. ($C_{23}H_{28}N_3O_2Br$) C, H, N.

10-(4'-*N*-Thiomorpholinobutyl)-2-bromoacridone (19)

Amounts of 1.11 g (3.05 mmol) of **11,** 1.45 g of KI, 2.42 g of K_2CO_3 and 1.3 mL (12.6 mmol) of thiomorpholine were refluxed and processed according to the procedure used for **10**. The oily residue was purified by column chromatography and treated with ethereal hydrochloride to give hydrochloride salt of compound **19** (yield 1.21 g, 55%, mp 226-230°C). UV λ_{max} (ε) 217(3,579), 256(8,430), 391(950), 410(186) nm; IR 3426, 2962, 1645, 1491, 1284, 836, 756, 615 cm^{-1}; ^{1}H-NMR δ 7.28-8.39 (m, Ar-H, 7H, H_l and H_3-H_8), 3.70-4.80 (m, 2H, H_k), 2.50-3.50 (m, 6H, H_a, H_b and H_n), 2.00 (m, 4H, H_c and H_d) and 1.87 (m, 4H, H_l and H_m); ^{13}C-NMR δ 127.21 (C_1), 122.52 (C_2), 125.22 (C_3), 118.71 (C_4), 116.23 (C_5), 134.76 (C_6), 121.31(C_7), 133.62 (C_8), 175.38 (C_9), 125.71($C_{1'}$), 140.31($C_{4'}$), 141.28($C_{5'}$), 121.53 ($C_{8'}$), 53.35 (C_k), 23.22 (C_l), 20.21(C_m), 42.77 (C_n), 53.21 (C_a and C_b) and 26.44 (C_c and C_d); MS m/z (%) 431[$M^{+\cdot}$,50].

Biological Activity

Determination of pK_a and Partition Coefficient

The pKa's were determined according to a previously published method [52]. The relative lipophilicity was assessed for each of the derivative using an adaptation of the method of Zamora *et al*. [53].

Cell Lines and Cell Culture

KB-3-1 and KBCh^R-8-5 Cells

Human KB epidermal carcinoma cells were subcloned two times; a single recloned cell line KB-3-1 has been used as the parent cell line for the present study. KBCh^R-8-5 cells were cross resistant to vincristine (45-fold) and VLB (~10-fold). KB-3-1 and KBCh^R-8-5 cells were grown in monolayer culture in antibiotic-free Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and L-glutamine in a humidified atmosphere of 5% CO_2 in air. The resistance of KBCh^R-8-5 cells was maintained by culturing in presence of 10 ng/mL of colchicine. KBCh^R-8-5 cells overexpressed *mdr1* and were positive for P-glycoprotein as determined by immunocytochemistry using HYB241 and C219 monoclonal antibodies.

Accumulation Studies

Two mL of cell suspension ($2x10^6$ cells) were plated in 35 x 10 mm style "easy grip" culture dishes (Greiner GmbH, Germany). Cells were allowed to attach to plastic overnight at 37 °C. Medium was aspirated and cells were washed with (2 x 2mL) PT buffer (120 mM NaCl, 20 mM Tris-base, 3 mM K_2HPO_4, 10 mM glucose, 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$, pH 7.4). Monolayers were incubated at room temperature for 10 minutes in PT buffer prior to aspiration

and adding 1mL of serum-free RPMI-1640 HEPES buffer (10.4g RPMI-1640 medium in 1 litre of 25 mM HEPES, pH 7.4) containing 49.9 nM [^{3}H] VLB (0.5 μCi/mL, specific activity 9.4 Ci/mmole; Amersham Pharmacia Biotech, UK, Ltd.,) with or without VRP (Sigma, St. Louis, MO) or acridone derivatives (**1-19**) at their respective IC_{50} concentrations, dissolved in DMSO (final culture concentration < 0.1% DMSO). After 2 hours of incubation at room temperature, medium was rapidly aspirated to terminate the drug accumulation and monolayers were washed four times with ice-cold PBS (g/L : NaCl 8.0; Na_2HPO_4, 12 H_2O 2.9 ; KCl 0.2; KH_2PO_4 0.2) buffer and drained. To each dish 1 mL of trypsin - EDTA (0.05% trypsin, 0.02% EDTA) was added. After 1 minute monolayers were triturated to give an uniform suspension of cells and radioactivity in 0.75 mL was determined by scintillation counting. Cell number per dish was determined using hemocytometer and amount of intracellular VLB was determined.

Measurement of VLB Efflux

Cells (2 x 10^6/dish) were plated and incubated overnight at 37°C in a CO_2 incubator to attach to plastic. Medium was removed and monolayer cells were washed once with 3 mL of PT buffer and incubated for 10 minutes in another 2 mL of the same buffer. KBCh^R-8-5 cells were incubated with 1mL of RPMI- 1640 (serum free) HEPES buffer, pH 7.4 containing 37.43 nM [^{3}H] VLB for 2 hours at room temperature. Drug solutions were aspirated and 1mL of the same buffer without or with modulator (**1-19**) at their respective IC_{50} concentrations or VRP at 100 μM were added and incubated for 2 more hours at room temperature. The medium was aspirated from each dish and the cells were washed four times in ice-cold PBS and drained. Cells were harvested and radioactivity per dish was calculated as described above.

Competition for [^{3}H] Azidopine Labeling of P-glycoprotein

Preparation of Plasma Membrane Fractions and Photoaffinity Labeling

Competition assay for photolabeling of P-gp used membranes from KB-V1 cells, which have higher P-gp levels than do KBCh^R-8-5 cells. Crude membranes were prepared from the MDR variant, KB-V1, essentially as described previously [54]. For photoaffinity labeling, membrane protein (200 μg) in buffer containing 250 mM sucrose and 10 mM Tris. HCl, pH 7.4 at 25°C, was mixed with 100 nM [^{3}H] azidopine (50 Ci/mmole; Amersham) in the absence or presence of modulators (1-19) at their IC_{50} concentration, in a total volume of 150 μL. After incubating for 20 minutes in the dark, the mixture was then irradiated with a germicidal UV-light (GE Germicidal Lights, G30T8, 30 Watts), commonly used in laminar flow hoods, for 20 minutes at a distance of 10-cm.

SDS - PAGE and Autoradiography

Photolabeled proteins were separated by one dimensional 5-15% SDS-PAGE under reducing conditions using the discontinous buffer system of Laemmli [55]. After staining with coomassie blue and destaining, the gels were soaked in Amplify (Amersham Corp.) for 30 minutes and dried under vaccum at 75 °C. The dried gels were exposed to film for two to three days at –70 °C and developed. Radioactivity labeled bands were scanned in their centres with a densiometer and quantified by integration with a chromatography recorder.

Inhibition of Cellular Growth

KB-3-1 or KBCh^R-8-5 at 5000 cells per well were plated in triplicate in 6 well flat bottom tissue culture plates (Greiner GmbH, Germany). After 24 hours at 37°C in an atmosphere of 5% CO_2-95% air, medium was replaced with 3 mL of fresh medium containing acridone compounds at concentration ranging from 0 - 100 μM in DMSO (final culture concentration < 0.1% DMSO) and cells were incubated at 37 °C for a further 7 days. The medium was aspirated and cells were washed twice with 1 mL of 0.9% saline. Colonies were stained with 1 mL of 0.1% crystal violet followed by washing with distilled water thrice and dried overnight. The IC_{10} and IC_{50} values were determined from concentration percent cell survival curves and were defined as the concentrations required for 10% and 50% reduction in colonies compared to controls.

Effect of N^{10}-substituted Acridones on *In Vitro* Cytotoxicity of VLB

To determine the effects of the modulators on the cytotoxicity of VLB, KB-3-1 or KBCh^R-8-5 cells were treated with serial dilutions of VLB (up to 100 nM for KBCh^R-8-5 and up to 20 nM for KB-3-1) in the absence or presence of IC_{10} concentration of modulators. After incubation for 7 days at 37°C, colonies were enumerated as described above. IC_{50} values were determined as previously described and the fold-potentiation was calculated from dividing the IC_{50} for VLB in the absence of modulator by the IC_{50} in the presence of modulating agent.

Isolation and Purification of Calmodulin from Bovine Testes

The protein has been isolated and purified according to the method described by Chafouleas *et al*. [56]. Briefly, approximately 1 kg of bovine testes was homogenized and centrifuged at 20 000 x *g* for 20 min at 4 °C. The pellet was re-extracted and centrifuged. The supernatant was subjected for heat treatment, followed by cooling the fluid rapidly to 5°C by immersing in ethanol–ice slurry. Centrifuged for 20 min at 20 000 x *g* at 4°C. The supernatant was then brought to a final concentration of 10 mM using imidazole buffer (pH 6.1) containing 1 mM EGTA and subjected for dialysis against the same buffer with several changes for 24 hours. The dialyzed protein solution was applied to a DEAE-cellulose column (1.5 x 40 cm), which was pre-equilibrated with 10 mM imidazole buffer containing 1 mM EGTA. The unbound protein was allowed to pass through the column, which was washed with the same buffer until the effluent had an optical density of 0.02 at 280 nm. A linear gradient (0–0.4 M NaCl) was then initiated with 30 mL/hour flow rate. Fractions (5 mL) were collected and protein concentration determined followed by simultaneous checking by SDS-PAGE (12%). Fractions showing the band in the region of 18 000 Da in SDS-PAGE were pooled and dialyzed

against 10 mM imidazole buffer containing 0.02% NaN_3 (pH 6.8). Protein was concentrated by filtering through ultra-membrane and the sample applied to a Sephadex G-100 column (1.5 x 77 cm) in the above buffer containing 100 mM NaCl. Five milliliters fractions were collected and protein concentration in each of the fraction was determined by Bradford method at 595 nm and calmodulin activity was assayed by the ability to stimulate phosphodiesterase. Peaks showing the activity were pooled, concentrated and total protein was estimated and used for all the experiments.

Validation of AMP Standard Curve

Freshly prepared standard solutions of AMP in the range 0.05–4.00 µg were analyzed by HPLC [Beckman model 334 gradient liquid chromatograph comprises a 421 micro-processor system controller, two model 110A single-piston reciprocating pumps, a dynamically stirred gradient mixing chamber, a model 210 sample injection valve, µ Bondapak ODS, 5 µ reverse phase steel column (4.6 x 15 cm) and a model 153 detector for UV detection at 254 nm, mobile phase: sodium phosphate buffer (50 mM, pH 6.4) containing 2% methanol, flow rate: 1 ml min^{-1}]. In the chromatogram, two peaks, one with retention time of 12 min for *c*AMP reference and the other with retention time of 4 min for AMP reference were obtained. A standard curve was obtained by plotting the peak height *versus* concentration, which was linear from 0.05 to 3.125 µg of AMP.

Phosphodiesterase Activity Assay

The assay mixture containing 25 mM Tris–HCl (pH 7.5), 25 mM imidazole (pH 7.5), 1.5 mM magnesium acetate, 2 µM Ca^{2+}, 1.25 x 10^{-4} U phosphodiesterase and 0.043 µmol of *c*AMP in a total of 0.15 mL, was incubated at 30 °C for 30 min (1U of enzyme activity is defined as the activity in the absence of calmodulin, which hydrolyzed 1.0 µmol of 3', 5'-cyclic AMP to 5'-AMP min^{-1} at pH 7.5). Reaction was terminated by heating the reaction mixture in a boiling water bath for 2 min. Denatured proteins were removed by centrifugation at 10 000 rpm for 10 min and 100 µl of supernatant was injected to HPLC. The amount of *c*AMP hydrolyzed by the enzyme was determined in terms of the amount of AMP formed, which could be estimated with the help of a previously obtained standard curve for AMP. Blank reactions were run concurrently with the test reaction for substrate blank correction.

Inhibition of Calmodulin-Dependent *c*AMP-Phospho-diesterase Activity by Acridones

Optimal assay conditions remain same as above. The assays were carried out in the presence and absence of different concentrations of potent acridone derivatives (**3, 4, 8, 9, 12**, **13, 17**, and **18**), in the range 0.001–100 µM, dissolved in DMSO. Before the reaction was started, 5 µL of modulator were added to the assay mixture. Addition of an equivalent amount of 5 µL of DMSO to the reaction mixture did not show any inhibitory effect on the calmodulin dependent *c*AMP-phosphodiesterase activity. The results are expressed as the concentration of inhibitor giving 50% inhibition of the calmodulin dependent *c*AMP-phospho-diesterase activity. The IC_{50} was determined from a plot of percentage activation *versus* varying concentrations of the modulator. To validate the phosphodiesterase assay method by HPLC, the IC_{50} value of 2-chlorpromazine was also determined.

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