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Original article

Anti-calmodulin acridone derivatives modulate vinblastine resistance in multidrug resistant (MDR) cancer cells

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Abstract

Multidrug resistance (MDR) is one of the main obstacles limiting the efficacy of chemotherapy treatment of tumors. Parent acridones **1A** and **1B** were prepared by the Ullmann reaction followed by cyclization and *N*-alkylation. *N*-(ω-Chloroalkyl) analogues were subjected to iodide catalyzed nucleophilic substitution reaction with secondary amines to get the compounds **3A–13A** and **3B–13B**, which enhanced the uptake of vinblastine in KBCh^R-8-5 cells to a greater extent (2.6–13.1-fold relative to control) than verapamil. The study on the structure–activity relationship revealed that substitution of –H at position C-4 in acridone nucleus by $-OCH_3$ 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.

Keywords: Acridones; Anti-MDR activity; Anti-calmodulin activity

1. Introduction

A significant problem in the clinical treatment of cancer arises from the development of tumor resistance to a wide variety of chemotherapeutic agents. The overexpression of drug transport proteins is a major mechanism for this multiple drug resistance (MDR) [1,2]. The drug transporter, P-glycoprotein (Pgp), is an ATP-dependent drug efflux pump whose biochemistry and pharmacology have been intensely studied. Pgp confers cross-resistance to a variety of structurally unrelated cytotoxic drugs, e.g. epipodophyllotoxins, vinca alkaloids, anthracyclines, taxanes and actinomycin D, and is able to efflux cytotoxic drugs against a concentration gradient, therefore, causing a cellular drug accumulation deficit, resulting in resistance. Recent studies showed that tumor cells expressing MDR-associated protein (MRP), lung resistant protein (LRP) and mutation of DNA topoisomerase II are likely to be MDR [3,4]. Because of the importance of MDR in clinical oncology, an intensive search for antagonists of these transporters has been undertaken since the demonstration that verapamil has this property [5]. Consequently, a variety of compounds have since been shown to reverse Pgp mediated MDR in vitro [6], and several of these agents have been evaluated in clinical trials [7,8]. Unfortunately, most of these agents suffer clinically from their intrinsic toxicity or from undesired effects on the pharmacokinetics of the accompanying anti-cancer drugs. These limitations have spurred on efforts to search for new and more effective compounds. Therefore, the development of potent Pgp inhibitors that are better tolerated, remains an important task in successfully overcoming MDR.

An acridonecarboxamide derivative [9] (GF120918) and 1,3-bis (9-oxoacridin-10-yl) propane [10] (a novel acridone) have already been shown to be among the group of compounds known to modify Pgp-mediated MDR. Initially the authors prepared the parent 2-chloro, 2-bromo, 2-methoxy and 4-methoxy acridones and evaluated for anti-MDR activity against KBCh^R-8-5 cells. Examination of the preliminary results revealed that a methoxy group at C-4 position exhib-

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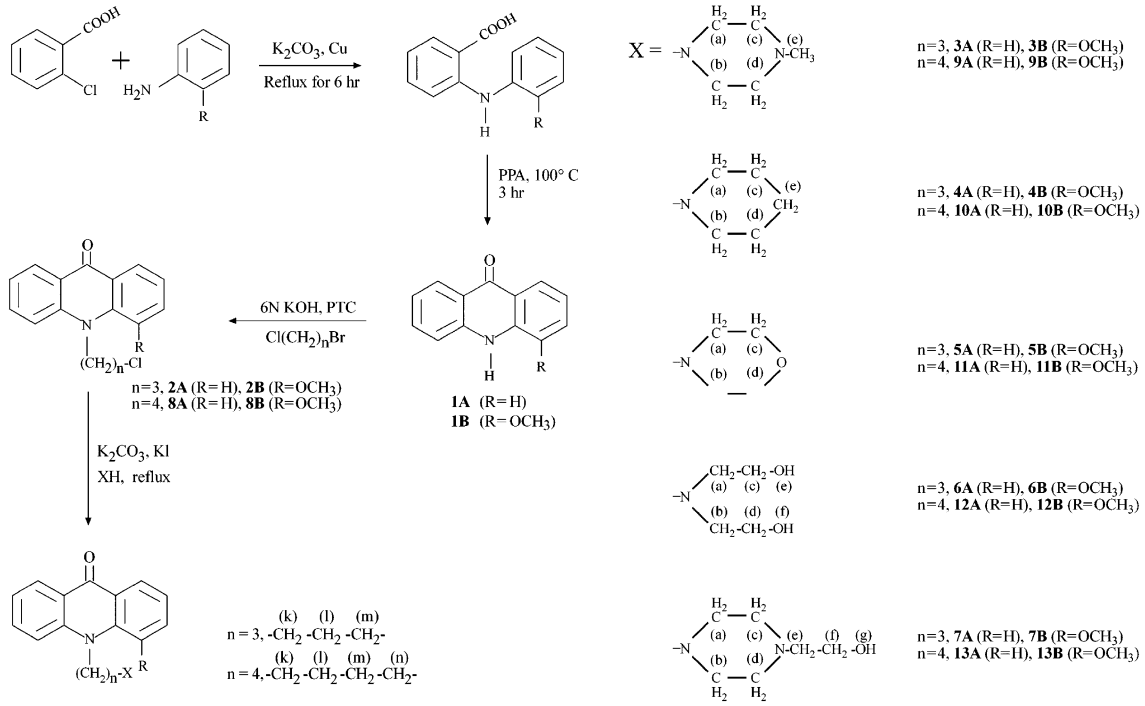


Scheme 1

ited the highest anti-MDR activity as compared to other substituents at C-2 position (unpublished data). Therefore, to extend the portfolio of better transporter antagonists, in this manuscript, the authors describe the synthesis and biological evaluation of a series of 26 N^{10}-substituted and 4-methoxy-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 these acridones with their effects on cellular growth and Pgp-mediated MDR.

2. Results and discussion

2.1. Chemistry

The ease of synthesis, attractive coloration, and crystallinity of acridine derivatives has attracted the attention of the medicinal chemists [11]. The basic tricyclic framework can be decorated with appropriate substituents to confer specificity against both prokaryotic and eukaryotic targets, which have given acridines a respectable reputation in the history of chemotherapy [12]. 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 [13–16]. In the present investigation, we have concentrated on developing simple synthetic routes together with improvements over published methods, and to give the first account of anti-MDR and anti-calmodulin activities of acridone derivatives.

The chemical synthesis of N^{10}-substituted (**1A–13A**) and 4-methoxy-N^{10}-substituted (**1B–13B**) acridones has been carried out according to Scheme 1. Ullman reaction of *o*-chlorobenzoic acid and aniline or anisidine gave diphenylamine-2-carboxylic acid or 2′-methoxydiphenylamine-2-carboxylic acid, which were then cyclized to **1A** or **1B** (62–64%) *plus* sulfonated acridones (38–36%) using sulfuric acid at 100 °C on a water-bath as evidenced by TLC. When cyclization was carried out with polyphosphoric acid, only single product of **1A** or **1B** with better yield (86% or 90%) was obtained.

Usually N^{10}-alkylation with alkyl halides is difficult due to the weakly basic nature of nitrogen of the acridone nucleus. However, it can be achieved in the presence of a strong base like sodium amide or sodium hydride under anhydrous conditions. The reaction of parent acridone (**1A** or **1B**) with chlorobromoalkanes in the presence of sodium amide in anhydrous toluene under reflux conditions gave respective N^{10}-(chloroalkyl)acridones. Besides requiring drastic experimental conditions, the N^{10}-alkylation using sodium amide resulted in a very low yield. To overcome this drawback, N^{10}-alkylation was carried out in the presence of a phase transfer catalyst (PTC), which is more easy to work with and gives better yield than the previously described method.

Stirring of acridones (**1A** or **1B**) at room temperature with $Br–(CH_2)_3–Cl$ 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) (**2A** and **2B**) or 10-(4′-chlorobutyl) (**8A** and **8B**) acridones in good yield. Ammonium salt transports hydroxide ion from aqueous phase to organic phase where the actual reaction takes place. The results are interpreted by deprotonation of the acridones (**1A** and **1B**) by OH^-, transferred by the catalyst into the organic layer. The anion formed may be regarded as a phenolate stabilized anion, which subsequently undergoes alkylation to form aromatized system.

Iodide catalyzed nucleophilic substitution of the N^{10}-chloropropyl or N^{10}-chlorobutyl acridone or -4-methoxyacridone with various secondary amines (*N*-methylpiperazine, piperidine, morpholine, ethanolamine and (β-hydroxyethyl) piperazine) by refluxing for different time intervals in the presence of potassium carbonate in anhydrous acetonitrile gave the free bases **3A–7A, 9A–13A** and **3B–7B, 9B–13B**.

All the products were separated and purified by column chromatography or recrystallization method and dried under high vacuum for more than 12 h. The purified compounds were characterized by UV, IR, ^{1}H- and ^{13}C-NMR and mass spectral methods and elemental analysis.

The UV spectral data of acridone derivatives are in close agreement with the spectral characteristics of analogous heterocyclic compounds [17,18].

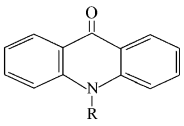
The IR spectra of parent compounds (**1A** and **1B**) and their derivatives (**2A–13A** and **2B–13B**) revealed the characteristic absorption of aromatic ketone and that of a secondary amino group. The strong bands in the region 1724–1610 cm^{-1} were assigned to >C=O stretching of the aromatic ring system and there is not much difference found in >C=O stretching frequency of the two series of acridone derivatives. Thus, the characteristic bands in the IR spectra support the proposed structure of acridones.

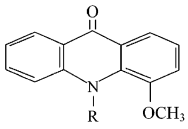
The ^{1}H-NMR spectrum of acridone showed eight aromatic protons at 'δ' 7.25–8.30 (m, H_1–H_8) for propyl and butyl derivatives (**1A–13A**). A singlet at 'δ' 11.80 was assigned to N–H proton of the aromatic ring. Similarly proton spectrum of 4-methoxyacridone showed seven aromatic protons at 'δ' 7.17–8.23 (m, H_1–H_3 and H_5–H_8) for propyl and butyl derivatives (**1B–13B**). Comparison of the ^{1}H-NMR spectral data of parent acridones (**1A** and **1B**) with those of corresponding N^{10}-substituted acridones showed clearly that chemical shift of all the common hydrogen atom slightly shifted to downfield (~0.2 ppm). The strong interaction between neighboring hydrogens (spin–spin coupling) which exists between *ortho*-hydrogens (J ~ 5–8 Hz) and weaker couplings by *meta*-hydrogens (J ~ 1–4 Hz) are very sensitive to substitution. Thus, a combination of chemical shift, spin–spin couplings and integration data permits the identification of individual hydrogen atoms at each side in the aromatic ring. The assignment of protons in all the compounds is fully supported by the integration curves and all the derivatives showed the characteristic chemical shifts for the acridone nucleus.

The ^{13}C-NMR spectrum of each of N^{10}-substituted series exhibited seven signals, whereas N^{10}-substituted-4-methoxyacridones showed 13 signals representing 13 magnetically different environmental aromatic carbons. Signal at 'δ' 176.62 in the spectrum of **1A** or at 'δ' 176.93 in the spectrum of **1B** was assigned to carbonyl carbon of the aromatic ring system. The chemical shift at the lower field was assigned to the carbon adjacent to carbonyl group probably due to deshielding effect offered by carbonyl oxygen. In the spectrum of **1A** at 'δ' 140.76 was assigned to $C_{4'}$ and $C_{10'}$ and it appeared in the downfield due to deshielding effect offered by ring nitrogen and signal at 'δ' 117.20 was assigned to C_4 and C_5 and it appeared in the upfield due to shielding effect offered by or the secondary nitrogen atom attached to *ortho* carbon. In the spectrum of **1B**, the signal at 'δ' 150.21 was assigned to $C_{10'}$ as it will experience maximum deshielding effect by the *ipso* secondary nitrogen. The signal at 'δ' 143.20 in the spectrum of **1B** was assigned to C_4 and is appeared in the downfield due to deshielding effect of oxygen in the 'OCH_3' group. The signals at 'δ' 122.05 and 20.82 were assigned to the bridged head carbons $C_{9'}$ and $C_{8'}$, respectively. The chemical shifts at 'δ' 123.71 and 127.27 were assigned to the *ortho* carbons (C_1 and C_8) and at 'δ' 119.23 and 135.07 to the *para* carbons (C_3 and C_6) to carbonyl carbon in the case of **1A**. Similar explanation for assigning the carbons holds good for rest of the compounds. In toto, ^{1}H- and ^{13}C-NMR spectral data were consistent with the proposed structures.

The mass spectral fragmentation characteristics of all the 26 acridones (**1A–13A, 1B–13B**) under electron ionization (EI) and electrospray ionization (ESI) conditions are studied. Although the basic structural unit is same, the difference in their structures arises because the substituents attached to N^{10}-position are of diverse functionality. They differ from each other because the terminal hydrogen of an alkyl group is replaced by either chlorine, *N*-methylpiperazine, piperidine, morpholine, diethanolamine or (β-hydroxyethyl) piperazine. In the EI mass spectra, fragmentation reactions are observed due to cleavage of bonds in the N^{10}-side chain portion and acridone ring system remains intact. This fact is manifested in the mass spectra of **1A–13A** and **1B–13B**. The preponderance of $[M + H]^+$ in the ESI spectra of **1A** and **1B** is a testimony to the stability of the acridone ring nucleus. No fragment ion peaks are observed in the spectra of parent acridones (**1A** and **1B**). Molecular ions were observed either in the form of $[M + H]^+$ or $[M]^{+\bullet}$ in the spectra of these acridone derivatives and molecular ion peaks are the base peak except for compounds **6A, 9A, 11A, 2B, 10B, 11B** and **12B**. From the mass spectral data, it is clear that as such there is no difference in fragmentation pattern among the two set of acridone series. In general, mass spectral features of these compounds are similar and straight forward. Most of the compounds yield abundant molecular ions in the form of $[M + H]^+$. All bonds in the N^{10}-side chain portion are prone to cleavage. In conclusion, the data presented here demonstrate the usefulness of MS for characterization of acridone derivatives.

Table 1
Effect of acridone derivatives on the accumulation of vinblastine in KBCh^{R}-8-5 cells





Comp. No.	R	pKa	$\log_{10}P^{a}$	VLB uptake (%control)[b]	Comp. No.	R	pKa	$\log_{10}P^{a}$	VLB uptake (%control)[b]
1A	-H	ND	1.85	255	1B	-H	ND	1.96	334
2A	-$(CH_2)_3$-Cl	7.80	1.93	350	2B	-$(CH_2)_3$-Cl	7.28	1.83	312
3A	-$(CH_2)_3$-N N-CH_3	4.90 7.87	1.35	619	3B	-$(CH_2)_3$-N N-CH_3	5.08 8.41	1.50	608
4A	-$(CH_2)_3$-N	4.90 8.01	1.46	656	4B	-$(CH_2)_3$-N	5.04 7.96	1.76	950
5A	-$(CH_2)_3$-N O	4.87 8.39	1.53	816	5B	-$(CH_2)_3$-N O	4.83 8.30	1.69	633
6A	-$(CH_2)_3$-N CH_2-CH_2-OH, CH_2-CH_2-OH	5.02 8.07	1.66	799	6B	-$(CH_2)_3$-N CH_2-CH_2-OH, CH_2-CH_2-OH	4.68 7.48	1.64	1219

Contd.

Table I Contd.

Comp. No.	R	pKa	$\log_{10}P^{a}$	VLB uptake (%control)[b]	Comp. No.	R	pKa	$\log_{10}P^{a}$	VLB uptake (%control)[b]
7A	-$(CH_2)_3$-N N-CH_2-CH_2-OH	5.06 8.21	1.38	602	7B	-$(CH_2)_3$-N N-CH_2-CH_2-OH	4.63 8.43	1.48	798
8A	-$(CH_2)_4$-Cl	7.78	1.83	422	8B	-$(CH_2)_4$-Cl	7.14	1.95	401
9A	-$(CH_2)_4$-N N-CH_3	5.15 8.45	1.46	680	9B	-$(CH_2)_4$-N N-CH_3	4.93 7.96	1.69	972
10A	-$(CH_2)_4$-N	5.73 7.98	1.76	989	10B	-$(CH_2)_4$-N	4.59 7.86	1.95	1226
11A	-$(CH_2)_4$-N O	4.95 7.40	1.55	694	11B	-$(CH_2)_4$-N O	4.93 8.18	1.78	796
12A	-$(CH_2)_4$-N CH_2-CH_2-OH, CH_2-CH_2-OH	4.50 8.25	1.74	884	12B	-$(CH_2)_4$-N CH_2-CH_2-OH, CH_2-CH_2-OH	4.46 7.95	1.91	931
13A	-$(CH_2)_4$-N N-CH_2-CH_2-OH	4.96 8.14	1.58	826	13B	-$(CH_2)_4$-N N-CH_2-CH_2-OH	4.47 7.83	1.90	1310

ND : Not determined
[a] Octanol / buffer partition coefficient
[b] $\left(\frac{\text{VLB uptake with modulator}}{\text{VLB uptake without modulator}}\right) \times 100$
Compounds were tested at 100μM. Each experiment was done in triplicate with a SD of less than 8% of the mean.

2.2. Biological activity

2.2.1. pK_a and lipophilicity

The efficacy of an MDR modulator will depend in part on its ability to accumulate in cells. The acridone derivatives are weak bases and able to exist in both charged (protonated) and uncharged (unprotonated) forms. The neutral or unprotonated form of compounds is able to diffuse freely and rapidly across biological membranes due to higher membrane permeability. 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 pK_a values and relative lipophilicity of acridone derivatives have been determined according to previously published methods [19,20].

The pK_a values of **1A–13A** and **1B–13B** lie in the range 7.14–8.45 (Table 1). For the parental molecules (**1A** and **1B**) and the four compounds substituted with a propyl or butyl bridge *plus* chlorine at N^{10}-position (**2A, 8A, 2B** and **8B**), there is a single pK_a near to physiologic pH (7.14–7.80). For the remaining derivatives with a tertiary amine substituted in place of chlorine, there are two pK_a values, one associated with the ring structure and the other associated with the

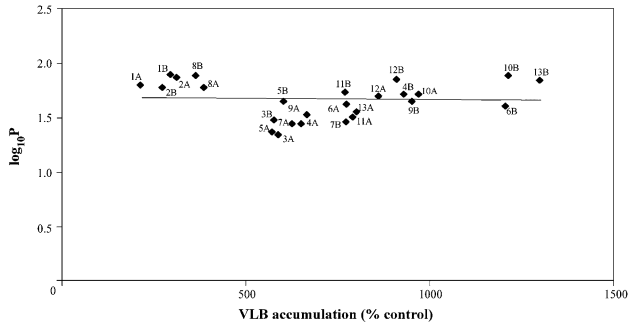


Fig. 1. Correlation between the $\log_{10} P$ of the acridones and their ability to enhance vinblastine accumulation in KBCh^R-8-5 cells. The N^{10}-substituted acridones are represented by **1A–13A** and N^{10}-substituted-4-methoxy-acridones are represented by **1B–13B**. The correlation coefficient was determined using the values from all derivatives.

tertiary amine. This substitution resulted in an increase in the pK_a associated with the acridone ring structure to values greater than 7.8 in all the cases except two compounds **11A** and **6B** (Table 1). This increase in basicity will result in the derivatives being uncharged at physiological pH. Thus, these derivatives will accumulate readily in cells as free bases, a result consistent with their increased ability to enhance vinblastine accumulation in an MDR cell line. The compounds without the tertiary amine (**1A, 2A, 8A, 1B, 2B** and **8B**) have pK_a closer to physiologic pH and therefore will accumulate more slowly in cells as the protonated form.

The lipophilicity data varying from 1.35 to 1.96, expressed in $\log_{10} P$, are given in Table 1. In order to elucidate the role played by the –OCH$_3$ group at position C-4 of the acridone ring, the lipophilicity data of **1B–13B** was compared with those of the corresponding unsubstituted acridone counterparts (**1A–13A**) where the position C-4 of the ring is occupied by a –H atom. In general, substitution of hydrogen by methoxy group in position C-4 of the acridone ring has resulted in a slight enhancement in the $\log_{10} P$ values. Additionally, it is speculated that the acridone nucleus with methoxy group at position C-4 may exhibit higher affinity for membranes or be more readily taken up into cells than that with a hydrogen atom.

Analysis of the relationship between $\log_{10} P$ values and the effectiveness of the modulators to increase vinblastine accumulation in KBCh^R-8-5 cells showed a poor correlation (Fig. 1, $R^2 = 0.0014$). There was a trend for the most lipophilic compounds (highest $\log_{10} P$ values) to be the most active for enhancing vinblastine accumulation. The major outlier in this analysis was the parent molecules (**1A** and **1B**) and the compounds without the tertiary amine (**2A, 8A, 2B** and **8B**) are comparatively having higher $\log_{10} P$ values than any of its substituted derivatives, yet these were not very effective at increasing vinblastine accumulation. In contrast, compound **10B** with the second highest $\log_{10} P$ value (1.95) was the compound, which significantly increased vinblastine accumulation (12.3-fold relative to control) in our model system. Therefore, the degree of lipophilicity of each drug would seem to be important, but it is not the sole determinant of potency for the Pgp-modulating activity of acridone derivatives.

*2.2.2. Effect of acridone derivatives (**1A–13A** and **1B–13B**) on cellular accumulation and the efflux of [^{3}H]vinblastine*

To identify the possible mechanism of potentiation of vinblastine by acridones, the effect of equimolar concentrations (100 μM) of these modifiers on the cellular accumulation of vinblastine in Pgp-mediated MDR KBCh^R-8-5 cells has been investigated and the results are shown in Table 1. After a 2-h incubation with 49.9 nM [^{3}H]vinblastine, all the 26 acridones exhibited a significant enhancing effect on vinblastine accumulation (2.6–13.1-fold relative to control) compared to a standard modulator verapamil (8.8-fold). Eighteen compounds (**1A–9A, 11A, 13A, 1B–3B, 5B, 7B, 8B** and **11B**) were found to possess less VLB enhancing effect (2.6–8.3-fold) compared to verapamil (8.8-fold) relative to control. Remaining eight compounds (**10A, 12A, 4B, 6B, 9B, 10B, 12B** and **13B**) caused a 1.0–1.5-fold greater accumulation of vinblastine than did a similar concentration of verapamil. The vinblastine accumulation enhancing effects with respect to percentage control are in the range 350–816% and 422–990% for propyl and butyl derivatives of unsubstituted acridone and 312–1219% and 401–1310% for propyl and butyl derivatives of 4-methoxyacridone, respectively, suggesting that the butyl derivatives seem to top the list on the vinblastine uptake enhancing effect in both the series of compounds. Among the 13 N^{10}-substituted acridones, only two compounds (**10A** and **12A**) enhanced the vinblastine accumulation greater than verapamil whereas out of 13 N^{10}-substituted-4-methoxyacridones six compounds (**4B, 6B, 9B, 10B, 12B** and **13B**) showed VLB uptake greater than verapamil suggesting that –OCH$_3$ substitution at C-4 of the acridone nucleus increased the efficacy to enhance the uptake of vinblastine in KBCh^R-8-5 cells by 1.0–1.5-fold. The greatest increase was for derivative **13B** (Table 1) and this was probably due to the enhanced lipophilicity of the compounds after –OCH$_3$ substitution. Additionally, it is speculated that the acridone nucleus with –OCH$_3$ group at position C-4 may exhibit a higher affinity for membranes or be more readily taken up into cells than that with a hydrogen atom. It is interesting to note that the parent acridones (**1A** and **1B**) were the least effective at increasing vinblastine uptake suggesting that N^{10}-substitution is required for optimal activity. Further, the accumulation data revealed that the ability of the unsubstituted and 4-methoxy acridones largely follow the order: N^{10}-butyl > N^{10}-propyl derivatives, although there are exceptions in the series.

In the efflux assay, the cell associated radiolabel remaining after 2 h was determined and calculated as a percentage of the vinblastine present after loading and the data are given in Table 2. The outward transport of vinblastine was very rapid and the intracellular level of vinblastine reached around 22% within 2 h in the absence of modulator. Whereas,

Table 2
Effect of acridone derivatives on the cellular retention of vinblastine in $KBCh^R$-8-5 cells after 2 h efflux

Compund number [a]	VLB retention (%)	Compound number [a]	VLB retention (%)
1A	30	**1B**	32
2A	36	**2B**	33
3A	59	**3B**	53
4A	43	**4B**	50
5A	45	**5B**	43
6A	70	**6B**	51
7A	55	**7B**	65
8A	32	**8B**	37
9A	74	**9B**	72
10A	50	**10B**	56
11A	74	**11B**	74
12A	40	**12B**	66
13A	65	**13B**	79
Control	22	Control	20
VRP	59	VRP	62

All values represent the mean of two separate experiments with a S.D. of less than 8% of the mean; each experiment was done in triplicate.
[a] Modulators used at 100 μM concentration.

30–79% of vinblastine in the presence of modulators or 59% of vinblastine in the presence of verapamil was retained in the cells. Nine (**6A, 9A, 11A, 13A, 7B, 9B** and **11B–13B**) out of 26 compounds exhibited greater efflux inhibiting activity than verapamil, suggesting that the acridones, like verapamil, are able to inhibit Pgp-mediated vinblastine efflux from $KBCh^R$-8-5 cells. Comparative account of the two series of compounds for their efficacy to inhibit vinblastine efflux suggests that 4-methoxyacridones (**1B–13B**) are more active than their counterparts (**1A–13A**).

2.2.3. In vitro cytotoxicities of acridones in drug sensitive and resistant cancer cell lines

The cytotoxicity of 26 compounds (**1A–13A** and **1B–13B**) was examined in $KBCh^R$-8-5 or GC_3/c1 by incubating cells with several concentrations (0.001, 0.01, 0.1, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 75.0 and 100.0 μM) of acridones and the results are given in Table 3. The IC_{10} and IC_{50} values for N^{10}-substituted acridones (**1A–13A**) lie, respectively, in the ranges 0.35–1.90 and 6.0–21.0 μM or for N^{10}-substituted-4-methoxyacridones (**1B–13B**) lie, respectively, in the ranges 0.35–4.1 and 6.0–19.0 μM for $KBCh^R$-8-5 cells. Compounds (**10A, 12A, 4B, 6B, 9B, 10B, 12B** and **13B**) which were effective in enhancing 2 h vinblastine accumulation were also the most cytotoxic (IC_{50} < 8 μM). Careful examination of IC_{50} values for N^{10}-substituted acridones or N^{10}-substituted-4-methoxyacridones against $KBCh^R$-8-5 cells revealed that cytotoxicity relatively increased as the –H atom at C-4 position is replaced by $–OCH_3$ group. In the case of GC_3/c1, the IC_{10} and IC_{50} values were determined for both the series of compounds. The IC_{50} values lie in the range 5.2–19.0 μM for N^{10}-substituted acridone derivatives or in the range 5.8–17.0 μM for N^{10}-substituted-4-methoxyacridones. It is very clear from this data that there is not much difference in IC_{50} values between the two series of compounds. Examination of the cytotoxic data of both the series of compounds against $KBCh^R$-8-5 and GC_3/c1 cells revealed that butyl derivatives were found to be more cytotoxic than their propyl counterparts suggesting that the cytotoxicity largely increased as the chain length increased from 3 to 4. Therefore, from this study we can tentatively conclude that the structural features required within the series to cause a maximum cytotoxic effect in $KBCh^R$-8-5 and GC_3/c1 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 cytotoxicity from these studies.

Acridones (**12A, 13A, 6B, 9B** and **11B–13B**) which completely reversed the resistance in $KBCh^R$-8-5 cells, were evaluated for cytotoxicity against the drug-sensitive (KB-3-1) cells. The IC_{10} values (data not given) and the respective

Table 3
Cytotoxicity of acridone derivatives in MDR cell lines

Compound number	$KBCh^R$-8-5 cells		GC_3/c1 cells		Compound number	$KBCh^R$-8-5 cells		GC_3/c1 cells	
	IC_{10} (μM)	IC_{50} (μM)	IC_{10} (μM)	IC_{50} (μM)		IC_{10} (μM)	IC_{50} (μM)	IC_{10} (μM)	IC_{50} (μM)
1A	1.90	21.00	2.23	18.50	**1B**	4.06	19.00	3.02	17.00
2A	1.50	19.01	1.36	18.02	**2B**	1.37	17.52	1.45	15.01
3A	0.90	12.00	1.95	13.10	**3B**	0.67	9.01	1.45	7.50
4A	0.95	9.04	1.58	13.00	**4B**	1.08	7.53	0.65	11.00
5A	0.80	10.05	2.05	12.00	**5B**	0.97	8.52	1.53	11.07
6A	1.81	11.00	1.52	9.00	**6B**	0.35	8.01	1.45	10.03
7A	0.88	9.42	1.55	11.02	**7B**	0.69	9.04	1.51	6.18
8A	1.05	17.07	3.83	19.00	**8B**	2.12	17.02	2.95	15.00
9A	0.74	10.51	1.12	9.05	**9B**	0.83	7.50	1.32	7.62
10A	0.83	7.50	0.98	7.11	**10B**	0.55	6.50	1.30	6.00
11A	0.75	7.00	0.85	6.02	**11B**	0.50	6.75	0.78	5.80
12A	0.65	8.01	1.05	5.20	**12B**	0.60	7.01	1.81	10.50
13A	0.35	6.00	1.90	9.50	**13B**	0.55	6.00	1.05	7.53

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 Section 3. 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.

Table 4
Effect of acridone modulators on the potentiation of vinblastine cytotoxicity in drug resistant KBCh^R-8-5 and GC$_3$/c1 cells

Compound number [a]	KBCh^R-8-5 cells		GC$_3$/c1 cells		Compound number [a]	KBCh^R-8-5 cells		GC$_3$/c1 cells	
	Vinblastine IC$_{50}$ (nM)	Fold-potentiation	Vinblastine IC$_{50}$ (nM)	Fold-potentiation		Vinblastine IC$_{50}$ (nM)	Fold-potentiation	Vinblastine IC$_{50}$ (nM)	Fold-potentiation
1A	22.5	3.0	6.1	1	**1B**	17.3	3.9	6.0	1.0
2A	18.1	3.8	5.8	1.1	**2B**	15.0	4.5	5.8	1.1
3A	5.5	12.3	1.0	6.1	**3B**	3.8	17.9	1.2	5.1
4A	9.2	7.4	2.9	2.1	**4B**	7.5	9.1	2.3	2.7
5A	4.8	14.2	2.2	2.8	**5B**	5.2	13.1	2.0	3.1
6A	2.9	23.4	1.3	4.7	**6B**	2.0	Complete [b]	0.9	6.8
7A	4.0	17.0	1.5	4.1	**7B**	3.8	17.9	1.1	5.5
8A	17.0	4.0	5.6	1.1	**8B**	14.2	4.8	5.6	1.1
9A	4.0	17.0	0.95	6.4	**9B**	2.1	Complete [b]	0.75	8.1
10A	7.0	9.7	1.2	5.1	**10B**	4.5	15.1	0.9	6.8
11A	4.2	16.2	1.7	3.6	**11B**	1.8	Complete [b]	1.7	3.6
12A	2.0	Complete [b]	0.8	7.6	**12B**	1.6	Complete [b]	5.2	1.2
13A	2.1	Complete [b]	1.2	5.1	**13B**	1.9	Complete [b]	0.7	8.7

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] Modulator used at the IC$_{10}$ concentration (Table 3).
[b] Complete reversal of vinblastine resistance.

IC$_{50}$ values of 4.5, 2.8, 4.0, 3.3, 2.7, 2.9 and 2.2 μM for **12A, 13A, 6B, 9B, 11B, 12B** and **13B** were obtained. The results demonstrate that KB-3-1 cells are more sensitive to acridones compared to KBCh^R-8-5 cells.

2.2.4. Sensitization of drug-resistant KBCh^R-8-5 and GC$_3$ /c1 cells by acridone derivatives

The two series of compounds (**1A–13A** and **1B–13B**) were evaluated for their ability to modulate the cytotoxicity of vinblastine in drug-sensitive KB-3-1 and its MDR subline KBCh^R-8-5 cell line and also in an unrelated drug-resistant cell line GC$_3$/c1. Cells (KB-3-1, KBCh^R-8-5 or GC$_3$/c1) were exposed continuously to 0–100 nM vinblastine for 7-days in the absence or presence of IC$_{10}$ concentrations of acridone modulators and the fold-potentiation of vinblastine cytotoxicity for KBCh^R-8-5 and GC$_3$/c1 cells are summarized in Table 4. The modulators enhanced the cytotoxicity of vinblasine by 3.0–42.5-fold and 1.0–8.7-fold against KBCh^R-8-5 and GC$_3$/c1 cells, respectively. Examination of the cytotoxicity data revealed that the modulator **12B** demonstrated to have the greatest effect followed by **11B, 13B, 6B ~ 12A, 9B ~ 13A** and so on. The derivatives (**12A, 13A, 6B, 9B** and **11B–13B**), like verapamil, were able to completely reverse the 25-fold resistance of KBCh^R-8-5 cells to vinblastine. The IC$_{50}$ values for continuous exposure to vinblastine was 2.7 nM in KB-3-1 and 68.0 nM in KBCh^R-8-5 cells in the absence of modulating agent. The most effective modulators (**12A, 13A, 6B, 9B, 11B, 12B** and **13B**) in KBCh^R-8-5 cells were subsequently tested in KB-3-1 and all were shown to cause a small sensitization (2.0–6.0-fold) of the drug-sensitive line to vinblastine. However, a similar degree of sensitization was also found when the classical MDR modulator, verapamil (3.8-fold) was used. Of interest was that the acridones are more effective in reversing MDR of KBCh^R-8-5 cells rather than GC$_3$/c1 cells. The MDR reversing ability of acridones with –OCH$_3$ at C-4 position is greater by 1.1–2.3-fold for KBCh^R-8-5 cells or 1.1–1.7-fold for GC$_3$/c1 cells when compared to their counterparts with hydrogen atom at C-4 position. The influence of alkyl bridge length between the ring nucleus and the amino group was examined. Increasing the length from three to four carbons increased the cytotoxic and anti-MDR activities.

Thus, the structural features required for significant reversal of Pgp-mediated MDR include a tricyclic hydrophobic acridone ring with a –OCH$_3$ group at position C-4 and a bishydroxyethylamino, *N*-methylpiperazino, morpholino or (β-hydroxyethyl)piperazino side chain containing a tertiary amino group at a distance of at least three or four carbon atoms from the 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 vinblastine accumulation (**12A, 6B, 9B, 12B** and **13B**) are among the compounds that can completely reverse vinblastine resistance in KBCh^R-8-5 cells. The relationship between fold-potentiation of vinblastine cytotoxicity in KBCh^R-8-5 and GC$_3$/c1 cells for **1A–13A** and **1B–13B** was examined and the '*R*' value of 0.58 for N^{10}-substituted acridones (**1A–13A**) and of 0.24 for N^{10}-substituted-4-methoxyacridones (**1B–13B**) showed lack of correlation (data not shown). As it is observed, there is no correlation between effects in one cell line versus the other, i.e. a modulator that may be good in KBCh^R-8-5 cells has little activity in GC$_3$/c1 cells, whereas modulators having reasonable activity in GC$_3$/c1 cells may not be the most effective in KBCh^R-8-5 cells. These results suggest that modulation appears to be a function of the nature of anti-cancer drug, type of tumor cells and the modulating agent. Further, examination of the relationship between hydrophobicity and antagonism of Pgp-mediated MDR in KBCh^R-8-5 cells (data not shown, $R^2 = 0.015$) or in GC$_3$/c1 cells (data not shown, $R^2 = 0.103$) showed no correlation.

Table 5
Inhibition of calmodulin dependent *c*AMP-phosphodiesterase (PDE) by acridone derivatives

Compound number	Inhibition of PDE activation IC_{50} (μM)	Compound number	Inhibition of PDE activation IC_{50} (μM)
1A	98.00	**1B**	95.00
2A	50.00	**2B**	45.00
3A	20.00	**3B**	16.00
4A	10.00	**4B**	8.00
5A	8.00	**5B**	6.00
6A	4.00	**6B**	3.00
7A	4.00	**7B**	3.00
8A	20.00	**8B**	18.00
9A	18.00	**9B**	17.00
10A	8.00	**10B**	5.00
11A	2.00	**11B**	2.00
12A	1.00	**12B**	1.00
13A	1.00	**13B**	0.50

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 Section 3. IC_{50} values are the mean of three experiments with a S.D. of less than 6% of the mean.

Thus, the degree of lipophilicity of each drug, although important, was not the sole determinant of potency for anti-MDR activity.

2.2.5. Correlation between anti-calmodulin activity and inhibition of cellular growth and MDR

It has been previously shown that calmodulin inhibitors are capable of significantly sensitizing MDR cells by possibly interfering with cellular drug accumulation [21,22]. To examine the role of calmodulin as a possible target for the effect of acridones on cellular growth and MDR, we have determined the IC_{50} values for the inhibition of calmodulin (Table 5) by acridones and compared with their IC_{50} values for the inhibition of cellular growth and 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. This 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 upto 3.13 μg ($R = 0.998$, Fig. 2). In the absence of added 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 91.00 nmol in the reaction mixture containing 1.25×10^{-4} U of phosphodiesterase. K_m of the enzyme for *c*AMP was found to be 4×10^{-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

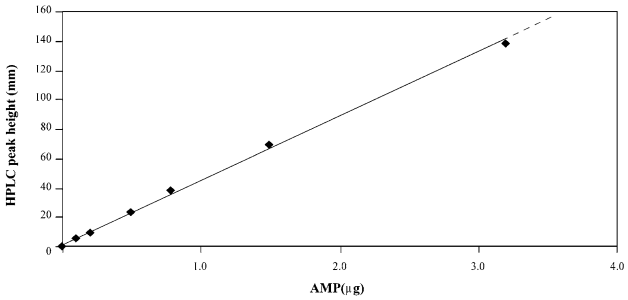


Fig. 2. A calibration curve for 5′-AMP. The concentration of 5′-AMP is expressed as HPLC peak height in millimeter. The curve is linear over the range investigated. Each point represents the mean of two experiments.

maximum enzyme activity, which closely approximates that found by others [23,24]. In order to determine the effect of calmodulin concentration for maximum phosphodiesterase activity, varied concentrations of activator upto 0.2 μg (0.0–1.0 U) were incubated with 1.24×10^{-4} U of phosphodiesterase containing 0.043 μmol of *c*AMP and the activity determined as described in Section 3. 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 6.6×10^{-3} μg (0.033 U) of activator. With saturating concentration of the activator, the enzyme activity was stimulated approximately by fivefold. 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 phosphodiesterase 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 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 1–98 μM for **1A–13A** or 0.5–95 μM for **1B–13B** compounds. Examination of the ability to inhibit calmodulin activity revealed that 4-methoxyacridones exhibited slightly higher potency than the unsubstituted counterparts. Comparison of the IC_{50} values within the series re-

vealed 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 **13B** (IC_{50} = 0.5 μM), **12B, 12A** and **13A** (IC_{50} of each = 1 μM), suggesting that the variation of the side chain is a strict requirement for activity. Replacement of bishydroxyethylamino or β-hydroxyethylpiperazino substituent by –Cl reduced the activity and introduction of a –H atom abolished it. Most of the potent calmodulin inhibitors [21,25–29] 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. Acridones 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 (Fig. 3A; R^2 = 0.71; $P < 0.0001$) or anti-MDR activity (Fig. 3B; R^2 = 0.43; $P = 0.0003$) for acridone derivatives are given. 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. These results suggest that inhibition of calmodulin, an intracellular calcium-binding protein that is known to play a key role in regulating cell proliferation [30], may be of importance in mediating the cytotoxic effects of acridones. Acridones have proven to be one of a number of diverse agents capable of reversing Pgp-mediated MDR [9,10]. Our comparative studies using two MDR cell lines, $KBCh^R$-8-5 and GC_3/c1 (shown to express Pgp) showed that acridones are at least as effective as verapamil in reversing resistance to vinblastine. 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. However, further mechanistic studies are necessary to elucidate the underlying basis of the cell line-specific antagonism of MDR.

In conclusion, our results indicate that combining a good calmodulin inhibitor with an anti-cancer compound might provide a strategy that could ultimately yield new modality for the treatment of refractory tumors.

3. Experimental

3.1. General

Reactions were monitored by TLC. Column Chromatography utilized silica gel Merck Grade 60 (230–400 mesh,

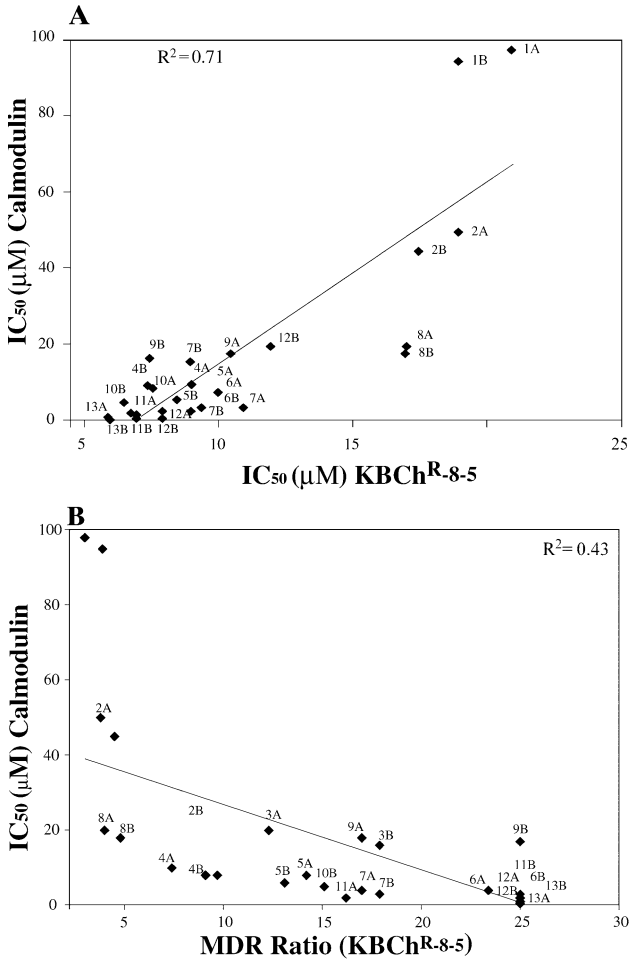


Fig. 3. (A) Correlation between the IC_{50} values (from Table 5) for inhibition of calmodulin-induced activation of phosphodiesterase and the IC_{50} values (from Table 3) for inhibition of $KBCh^R$-8-5 cellular growth for acridone derivatives (**1A–13A** and **1B–13B**). The coefficient of determination, R^2 = 0.71. The two-tailed *P*-value is <0.0001, considered extremely significant. (B) Correlation between the IC_{50} values (from Table 5) for inhibition of calmodulin-induced activation of phosphodiesterase and anti-MDR activity ($KBCh^R$-8-5 cells) for **1A–13A** and **1B–13B**. MDR ratios are from Table 4. R^2 = 0.43. The two-tailed *P*-value is 0.0003, considered extremely significant.

60 Å). 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 spectrometer as KBr pellets. Elemental analyses were performed and found values are within 0.4% of theoretical values unless otherwise noted.

^{1}H- and ^{13}C-NMR spectra were recorded in DMSO solution in a 5-mm tube on a Bruker drx 500 Fourier transform spectrometer with tetramethylsilane as internal standard. Chemical shifts are expressed as δ (ppm) values. The spectrometer was internally locked to deuterium frequency of the solvent. To obtain molecular weight information, acridone derivatives were analyzed by ESI and EI mass spectrometry. In ESI, the sample solution is injected into a continuous stream of solution that consists of 1:1 mixture of 0.1% trifluoroacetic acid and acetonitrile. In this study, a single

quadrupole mass spectrometer (platform II, micromass) was employed to analyze acridones. ESI is a gentle mode of ionization. It mainly produces protonated ions of the analyte molecules and no fragmentation provided the sampling cone voltage <30 V. In order to reduce the thermally induced fragmentation, the temperature of the ion source was adjusted to 60 °C. The quadrapole was scanned in the mass range of 50–450 Da. The EI technique used an AutoSpec Q (VG Analytical, Manchester, UK) hybrid tandem mass spectrometer of E_1BE_2-qQ geometry (where E is an electric sector, B a magnetic sector, q an rf-only quadrapole and Q a quadrapole mass analyzer). Only the front end (i.e. E_1BE_2) was used for this study. The data accumulation and manipulation were done under digital Vax Station 3100-based Opus Software. EI-ionizing energy, 70 eV; emission current, 100 μA; source temperature, 100 °C; mass resolution 2000; accelerating potential, 8000 V, mass range 50–500 Da; spectral scan rate, 5 s per decade; samples were introduced via direct insertion probe, which was not heated; the only source of it to the sample was through contact with the ion source.

3.2. Materials

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 form 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).

3.3. Chemistry

3.3.1. Preparation of diphenylamine-2-carboxylic acid

3.3.1.1. Ullmann condensation. To a mixture of *o*-chlorobenzoic acid (10 g, 0.064 mol), aniline (5.84 ml, 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 allowed to reflux for 6 h on an oil bath. The isoamylalcohol was removed by steam distillation and the mixture poured into 1 l 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 80%), m.p. 182 °C.

*3.3.1.2. Cyclization of diphenylamine-2-carboxylic acid to acridone (**1A**).* Nine grams of diphenylamine-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 °C for 3 h. Appearance of yellow color indicated the completion of the reaction. Then, it was poured into 1 l of hot water and made alkaline by liquor ammonia and the yellow precipitate formed was filtered, washed with hot water and collected. The sample of 9(10-H) acridone (**1A**) was recrystallized from acetic acid (yield 86%). M.p. 353 °C, UV λ_{max} (ε) (MeOH) 214 (47 683), 254 (99 939), 380 (14 573) and 398 (15 000) nm; IR: 3280, 3100, 2915, 2855, 1632, 1600, 1550, 1473, 1350, 1272, 1168, 1020, 930, 758, 678 and 555 cm^{-1}; ^{1}H-NMR: δ 7.25–8.30 (m, Ar-H, 8H, H_1–H_8) and 11.8 (s, NH); ^{13}C-NMR: δ 176.62 (C_9), 140.76 ($C_{4'}$ and $C_{10'}$), 133.29 (C_3 and C_6), 125.89 (C_1 and C_8), 120.85 ($C_{8'}$ and $C_{9'}$), 120.37 (C_2 and C_7) and 117.20 (C_4 and C_5); MS: *m/z* 196 ([M + H]$^+$, 100). Anal. ($C_{13}H_9NO$) C, H, N.

3.3.2. Synthesis of N^{10}-alkylated acridones via phase transfer catalysis

*3.3.2.1. 10-(3′-Chloropropyl)acridone (**2A**).* Five grams (0.026 mol) of acridone was dissolved in 100 ml tetrahydrofuran and then 80 ml of 6 N potassium hydroxide and 2 g (0.006 mol) of tetrabutylammonium bromide was added to it. The reaction mixture was stirred at room temperature for 30 min and added 1-bromo-3-chloropropane (0.065 mol) slowly into the reaction mixture and stirred for 24 h at room temperature. Tetrahydrofuran was evaporated and the aqueous layer was 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 chromatography by using the solvent system chloroform/acetone (8:1) to give a yellow solid of 10-(3′-chloropropyl)acridone (**2A**) (yield 48%). M.p. 183 °C, UV λ_{max} (ε) (MeOH) 216 (25 806), 254 (48 968), 383 (7419) and 402 (8194) nm; IR: 3504, 2919, 1724, 1591, 1558, 1500, 1343, 1263, 1180, 948, 753, 673 and 544 cm^{-1}; ^{1}H-NMR: δ 7.30–8.38 (m, Ar-H, 8H, H_l–H_8), 3.94–3.97 (t, 2H, H_m), 3.67–3.71 (t, 2H, H_K) and 2.26–2.30 (m, 2H, H_l); ^{13}C-NMR: δ 176.48 (C_9), 141.42 ($C_{4'}$ and $C_{10'}$), 134.26 (C_3 and C_6), 126.82 (C_1 and C_8), 121.66 ($C_{8'}$ and $C_{9'}$), 121.33 (C_2 and C_7), 115.45 (C_4 and C_5), 42.69 (C_k), 42.94 (C_m) and 29.52 (C_l); MS: *m/z* 272 ([M + H]$^+$, 100). Anal. ($C_{16}H_{14}ClNO$) C, H, N.

*3.3.2.2. 10-[3′-*N*-(Methylpiperazino)propyl] acridone (**3A**).* One gram (3.68 mmol) of 10-(3′-chloropropyl) acridone was dissolved in 60 ml of anhydrous acetonitrile and 1.48 g potassium iodide and 2.3 g of potassium carbonate were added and refluxed for 30 min. Then added 1 ml (9.1 mmol) of *N*-methylpiperazine into it slowly and refluxed for 15 h

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 using the solvent system chloroform/acetone (8:1) to give a light yellow oil of 10-[3′-*N*-(methylpiperazino)propyl] acridone (**3A**). An acetone solution of the free base was treated with ethereal hydrochloride to give the hydrochloride salt, which was dried over high vacuum to get pure solid (**3A**) (yield 58%). M.p. 133–135 °C), UV λ_{max} (ε) (MeOH) 217 (26 747), 256 (51 687), 386 (7831) and 404 (9036) nm; IR: 3384, 2982, 2420, 1625, 1591, 1540, 1500, 1465, 1375, 1271, 1180, 958, 768, 754 and 663 $cm^{-1;}$ ^{1}H-NMR: δ 7.45–8.48 (m, 8H, Ar-H, H_1–H_8), 2.59–2.63 (m, 12H, H_a, H_b, H_c, H_d, H_k and H_m), 2.50 (s, 3H, H_e) and 2.30–2.40 (m, 2H, H_l); ^{13}C-NMR: δ 176.59 (C_9), 141.33 ($C_{4'}$ and $C_{10'}$), 134.40 (C_3 and C_6), 126.79 (C_1 and C_8), 121.59 ($C_{8'}$ and $C_{9'}$), 121.49 (C_2 and C_7), 115.94 (C_4 and C_5), 42.65 (C_m), 42.07 (C_k), 49.28 (C_c and C_d), 48.01 (C_a and C_b), 30.67 (C_e) and 21.37 (C_l); MS: *m/z* 335 ($[M]^{+\bullet}$, 100).

*3.3.2.3. 10-(3′-*N*-Piperidinopropyl) acridone (**4A**).* The procedure used for **3A** was repeated with 1.13 g (4.16 mmol) of **2A**, 1.7 g of KI, 2.6 g of K_2CO_3 and 1.2 ml (12.82 mmol) of piperidine. The product was purified by column chromatography to give a yellow oily product which was converted into hydrochloride salt **4A** (yield 47%, m.p. not determined due to hygroscopic nature). UV λ_{max} (ε) (MeOH) 216 (24 357), 255 (37 928), 382 (5964) and 400 (6094) nm; IR: 3412, 2863, 1635, 1605, 1490, 1460, 1377, 1290, 1268, 1208, 1176, 1102, 937, 768, 674 and 570 cm^{-1}; ^{1}H-NMR: δ 7.45–8.48 (m, 8H, Ar-H, H_1–H_8), 2.59–2.63 (m, 12H, H_a, H_b, H_c, H_d, H_k and H_m), 2.50 (s, 2H, H_e) and 2.30–2.40 (m, 2H, H_l); ^{13}C-NMR: δ 176.12 (C_9), 134.39 ($C_{4'}$ and $C_{10'}$), 131.61 (C_3 and C_6), 128.67 (C_1 and C_8), 126.80 ($C_{8'}$ and $C_{9'}$), 121.48 (C_2 and C_7), 116.02 (C_4 and C_5), 57.63 (C_k), 52.95 (C_m), 51.98 (C_a and C_b), 23.28 (C_c and C_d), 23.10 (C_e) and 19.21 (C_l); MS: *m/z* 321 ($[M + H]^+$, 100).

*3.3.2.4. 10-(3′-*N*-Morpholinopropyl) acridone (**5A**).* Repeated the procedure used for **3A** with 1.08 g (3.98 mmol) of **2A**, 1.6 g of KI, 2.5 g of K_2CO_3 and 1.04 ml (11.94 mmol) of morpholine. The product was purified by column chromatography and converted into hydrochloride salt of **5A** (yield 40%, m.p. not determined due to hygroscopic nature). UV λ_{max} (ε) (MeOH) 216 (23 445), 256 (56 670), 385 (7075) and 402 (11 260) nm; IR: 3472, 2934, 2680, 1644, 1473, 1440, 1408, 1376, 1312, 1265, 1205, 1147, 1084, 989, 963, 896, 767, 636, 610 and 499 cm^{-1}; ^{1}H-NMR: δ 7.35–8.37 (m, 8H, Ar-H, H_1–H_8), 3.22–3.28 (m, 4H, H_c and H_d), 2.50 (t, 4H, H_a and H_b), 2.26 (m, 4H, H_k and H_m) and 1.08–1.09 (t, 2H, H_l); ^{13}C-NMR: δ 179.46 (C_9), 141.84 ($C_{4'}$ and $C_{10'}$), 136.44 (C_3 and C_6), 127.40 (C_1 and C_8), 121.60 ($C_{8'}$ and $C_{9'}$), 121.47 (C_2 and C_7), 116.17 (C_4 and C_5), 62.48 (C_c and C_d), 51.12 (C_k), 43.81 (C_m), 42.08 (C_a and C_b) and 21.98 (C_l); MS: *m/z* 323 ($[M + H]^+$, 100).

*3.3.2.5. 10-(3′-*N*-[Bis[hydroxyethyl]amino]propyl) acridone (**6A**).* One gram (3.68 mmol) of **2A**, 1.48 g of KI, 2.3 g of K_2CO_3 and 0.7 ml (7.36 mmol) of diethanolamine were refluxed for 15 h and followed rest of the procedure used for **3A**. Purification by column chromatography afforded a light yellow solid of **6A** (yield 56%). M.p. 118–120 °C, UV λ_{max} (ε) (MeOH) 216 (30 000), 255 (61 447), 386 (9659) and 402 (10 681) nm; IR: 3384, 2920, 2811, 1632, 1594, 1565, 1501, 1460, 1382, 1272, 1182, 1030, 930, 753 and 674 cm^{-1}; ^{1}H-NMR: δ 7.33–8.38 (m, 8H, Ar-H, H_1–H_8), 3.55 (t, 4H, H_c and H_d), 2.71–2.73 (t, 4H, H_a and H_b), 2.59–2.62 (t, 4H, H_k and H_m), 2.51 (s, 2H, H_e and H_f, disappearing on D_2O exchange) and 1.91 (p, 2H, H_l); ^{13}C-NMR: δ 176.49 (C_9), 141.51 ($C_{4'}$ and $C_{10'}$), 134.17 (C_3 and C_6), 126.70 (C_1 and C_8), 121.59 ($C_{8'}$ and $C_{9'}$), 121.51 (C_2 and C_7), 115.98 (C_4 and C_5), 59.30 (C_c and C_d), 56.83 (C_a and C_b), 52.07 (C_k), 43.79 (C_m) and 24.86 (C_l); MS: *m/z* 341 ($[M + H]^+$, 87). Anal. ($C_{20}H_{24}N_2O_3$) C, H, N.

*3.3.2.6. 10-(3′-*N*-[(β-Hydroxyethyl)piperazino] propyl) acridone (**7A**).* Amounts of 1.21 g (4.46 mmol) of **2A**, 1.7 g of KI, 2.7 g of K_2CO_3 and 1.35 ml (9.8 mmol) of (β-hydroxyethyl)piperazine were refluxed and processed according to the experimental conditions for **3A**. The oily residue was purified by column chromatography and treated with ethereal hydrochloride to give hydrochloride salt of **7A** (yield 51%). M.p. 140–142 °C, UV λ_{max} (ε) (MeOH) 216 (10 310), 255 (54 068), 386 (8851) and 404 (10 310) nm; IR: 3480, 2928, 2672, 2541, 1648, 1456, 1420, 1294, 1190, 1163, 1052, 962, 918, 752, 667 and 462 cm^{-1}; ^{1}H-NMR: δ 7.30–8.27 (m, Ar-H, 8H, H_1–H_8), 4.53 (t, 1H, H_g), 4.03 (t, 2H, H_f), 3.10–3.70 (m, 14H, H_a, H_b, H_c, H_d, H_e, H_k and H_m) and 1.70–1.95 (m, 2H, H_l); ^{13}C-NMR: δ 177.04 (C_9), 144.21 ($C_{4'}$ and $C_{10'}$), 134.33 (C_3 and C_6), 126.30 (C_1 and C_8), 122.51 ($C_{8'}$ and $C_{9'}$), 121.77 (C_2 and C_7), 116.45 (C_4 and C_5), 56.79 (C_f), 53.63 (C_e), 53.05 (C_k), 51.05 (C_m), 46.31 (C_a and C_b), 46.20 (C_c and C_d) and 22.63 (C_l); MS: *m/z* 366 ($[M + H]^+$, 100). Anal. ($C_{22}H_{27}O_2N_3$) C, H, N.

*3.3.2.7. 10-(4′-Chlorobutyl) acridone (**8A**).* Compound **8A** (yield 60%) in the pure form was synthesized following the procedure used for **2A** with 5.12 g (0.026 mmol) of **1A**, 2.0 g (0.006 mmol) of tetrabutylammonium bromide and 1-bromo-4-chlorobutane (0.065 mol). The crude product was purified by column chromatography. M.p. 134 °C, UV λ_{max} (ε) (MeOH) 216 (15 324), 256 (31 465), 386 (5183), 405 (5972) nm; IR: 3420, 2934, 1632, 1591, 1494, 1459, 1377, 1291, 1264, 1172, 1038, 937, 755, 674 and 544 cm^{-1}; ^{1}H-NMR: δ 7.33–8.38 (m, Ar-H, 8H, H_1–H_8), 3.66–3.69 (t, 2H, H_n), 2.51–2.52 (t, 2H, H_k) and 1.89–2.13 (m, 4H, H_l and H_m); ^{13}C-NMR: δ 176.44 (C_9), 142.30 ($C_{4'}$ and $C_{10'}$), 135.00 (C_3 and C_6), 126.88 (C_1 and C_8), 120.87 ($C_{8'}$ and $C_{9'}$), 119.98 (C_2 and C_7), 113.98 (C_4 and C_5), 51.37 (C_n), 45.83 (C_k), 23.94

(C_l) and 22.41 (C_n); MS: *m/z* 286 ([M + H]$^+$, 100). Anal. ($C_{17}H_{16}ClNO$) C, H, N.

*3.3.2.8. 10-(4′-*N*-(Methylpiperazino)butyl) acridone (**9A**).* Amounts of 1.15 g (4.03 mmol) of **8A**, 1.7 g of KI, 2.6 g of K_2CO_3 and 0.98 ml (8.9 mmol) of *N*-methylpiperazine in 60 ml of acetonitrile were refluxed for 15 h and worked up according to protocol used for **3A**. The product was purified by column chromatography and the free base in the form of a yellow solid (yield 55%) was obtained. M.p. 136 °C, UV λ_{max} (ε) (MeOH) 216 (11 955), 256 (22 799), 386 (3429), 402 (3985) nm; IR: 3422, 2937, 2785, 1634, 1600, 1495, 1461, 1382, 1294, 1268, 1181, 1162, 1042, 937, 794, 754 and 674 cm^{-1}; ^{1}H-NMR: δ 7.33–8.40 (m, Ar–H, 8H, H_1–H_8), 2.39–2.43 (m, 8H, H_a, H_b, H_c and H_d), 2.18 (s, 3H, H_e), 1.70–1.76 (m, 4H, H_k and H_n) and 1.22–1.38 (m, 4H, H_l and H_m); ^{13}C-NMR: δ 176.44 (C_9), 141.45 ($C_{4'}$ and $C_{10'}$), 134.12 (C_3 and C_6), 126.67 (C_1 and C_8), 121.55 ($C_{8'}$ and $C_{9'}$), 121.14 (C_2 and C_4), 115.96 (C_4 and C_5), 57.57 (C_n), 54.81 (C_k), 45.73 (C_a and C_b), 45.00 (C_c and C_d), 40.12 (C_e), 23.05 (C_l) and 19.15 (C_n); MS: *m/z* 350 [M + H]$^+$.

*3.3.2.9. 10-(4′-*N*-Piperidinobutyl) acridone (**10A**).* The experimental steps used for **4A** were repeated by taking 1.02 g (3.57 mmol) of **8A**, 1.5 g of KI, 2.3 g of K_2CO_3 and 0.88 ml (8.9 mmol) of piperidine. The oily product was purified by column chromatography and then converted into hydrochloride salt of **10A** (yield 45%). M.p. 153–155 °C, UV λ_{max} (ε) (MeOH) 215 (31 875), 254 (60 625), 380 (8000) and 399 (8625) nm; IR: 3442, 2938, 2762, 1643, 1597, 1493, 1420, 1376, 1281, 1158, 1036, 943, 782, 668 and 596 cm^{-1}; ^{1}H-NMR: δ 7.34–8.38 (m, Ar–H, 8H, H_1–H_8), 3.05–3.20 (m, 8H, H_k, H_n, H_a and H_b), 1.51–2.1 (m, 8H, H_l, H_m, H_c and H_d) and 1.22–1.45 (m, 2H, H_e); ^{13}C-NMR: δ 176.47 (C_9), 141.40 ($C_{4'}$ and $C_{10'}$), 134.28 (C_3 and C_6), 126.76 (C_1 and C_8), 121.60 ($C_{8'}$ and $C_{9'}$), 121.31 (C_2 and C_7), 115.92 (C_4 and C_5), 55.35 (C_k), 51.87 (C_a and C_b), 44.89 (C_n), 23.10 (C_l), 22.13 (C_m), 21.46 (C_c and C_d) and 20.29 (C_e); MS: *m/z* 336 [M + 2H]$^+$. Anal. ($C_{22}H_{26}N_2O$) C, H, N.

*3.3.2.10. 10-(4′-*N*-Morpholinobutyl) acridone (**11A**).* The method employed for **5A** was used with 1.10 g (3.85 mmol) of **8A**, 1.6 g of KI, 2.5 g of K_2CO_3 and 0.94 ml (10.78 mmol) of morpholine. The oily product was purified by column chromatography. Light colored oil obtained was converted into hydrochloride salt (yield 43%). M.p. 141–142 °C, UV λ_{max} (ε) (MeOH) 216 (43 882), 256 (95 529), 386 (15 647) and 405 (17 765) nm; IR: 3407, 2961, 2871, 2459, 1694, 1618, 1489, 1455, 1364, 1272, 1091, 876 and 758 cm^{-1}; ^{1}H-NMR: δ 7.35–8.40 (m, Ar–H, 8H, H_1–H_8), 3.15–3.20 (t, 4H, H_c and H_d), 2.18 (t, 4H, H_a and H_b), 2.10 (t, 4H, H_k and H_n) and 1.21–1.35 (m, 4H, H_l and H_m); ^{13}C-NMR: δ 176.78 (C_9), 140.90 ($C_{4'}$ and $C_{10'}$), 133.40 (C_3 and C_6), 125.98 (C_1 and C_8), 120.96 ($C_{8'}$ and $C_{9'}$), 120.50 (C_2 and C_7), 117.31 (C_4 and C_5), 61.50 (C_c and C_d), 58.31 (C_k), 53.18 (C_n), 40.19 (C_a and C_b), 23.09 (C_l) and 20.79 (C_m); MS: *m/z* 336.6 [M + H]$^+$.

*3.3.2.11. 10-(4′-*N*-[Bis[hydroxyethyl]amino]butyl) acridone (**12A**).* The steps used for **6A** were repeated with 1 g (3.68 mmol) of **8A**, 1.48 g of KI, 2.3 g of K_2CO_3 and 0.77 ml (8.1 mmol) of diethanolamine. The product was purified by column chromatography to give a pale yellow solid of **12A** (yield 60%). M.p. 143 °C, UV λ_{max} (ε) (MeOH) 216 (32 982), 256 (70 936), 380 (8561) and 399 (9123) nm; IR: 3384, 2944, 2800, 1648, 1614, 1561, 1505, 1462, 1392, 1293, 1273, 1181, 1061, 840, 748, 674 and 610 cm^{-1}; ^{1}H-NMR: δ 7.33–8.37 (m, Ar–H, 8H, H_1–H_8), 3.46–3.49 (t, 4H, H_c and H_d), 2.55–2.61 (m, 8H, H_k, H_n, H_a and H_b), 2.51 (s, 2H, H_e and H_f, disappearing on D_2O exchange), 1.78–1.88 (p, 2H, H_m) and 1.65–1.74 (p, 2H, H_l); ^{13}C-NMR: δ 176.45 (C_9), 141.43 ($C_{4'}$ and $C_{10'}$), 134.14 (C_3 and C_6), 126.71 (C_1 and C_8), 121.58 ($C_{8'}$ and $C_{9'}$), 121.14 (C_2 and C_4), 115.93 (C_4 and C_5), 59.36 (C_c and C_d), 56.56 (C_k), 54.17 (C_n), 45.32 (C_a and C_b), 24.23 (C_l) and 23.73 (C_m); MS: *m/z* 355 ([M + H]$^+$, 100). Anal. ($C_{21}H_{26}N_2O_3$) C, H, N.

*3.3.2.12. 10-(4′-*N*-([β-Hydroxyethyl]piperazino)butyl) acridone (**13A**).* Compound **13A** as its hydrochloride salt (yield 54%) was obtained by following the protocol of **7A** with 1.2 g (4.2 mmol) of **8A**, 1.8 g of KI, 2.8 g of K_2CO_3 and 1.13 ml (9.34 mmol) of β-hydroxyethyl piperazine. The product was purified by column chromatography. M.p. 196–198 °C, UV λ_{max} (ε) (MeOH) 216 (29 875), 256 (65 375), 380 (8185) and 399 (9625); IR: 3448, 2921, 2643, 2438, 1631, 1578, 1444, 1413, 1311, 1245, 1189, 1130, 1072, 990, 876, 816, 658 and 539 cm^{-1}; ^{1}H-NMR: δ 7.30–8.28 (m, Ar–H, 8H, H_1–H_8), 4.51–4.55 (t, 1H, H_g), 4.02 (s, 2H, H_f), 3.40–3.75 (m, 14H, H_a, H_b, H_c, H_d, H_e, H_k and H_n) and 1.70–2.05 (m, 4H, H_l and H_m); ^{13}C-NMR: δ 176.74 (C_9), 144.29 ($C_{4'}$ and $C_{10'}$), 134.11 (C_3 and C_6), 126.35 (C_1 and C_8), 122.34 ($C_{8'}$ and $C_{9'}$), 121.62 (C_2 and C_7), 116.47 (C_4 and C_5), 56.90 (C_f), 51.24 (C_e), 49.22 (C_k), 48.00 (C_n), 41.12 (C_c and C_d), 40.16 (C_a and C_b), 26.75 (C_l) and 20.79 (C_m); MS: *m/z* 380 ([M + H]$^+$, 100).

*3.3.3. Synthesis of 4-methoxy-N^{10}-substituted acridones (**1B–13B**)*

3.3.3.1. 2′-Methoxydiphenylamine-2-carboxylic acid. 2′-Methoxydiphenylamine-2-carboxylic acid as a greenish solid (yield 83%, m.p. 176 °C) was prepared by following the protocol used for diphenylamine-2-carboxylic acid with 15 g of *o*-chlorobenzoic acid (0.096 mmol), 11.74 g of *o*-anisidine (10.096 mmol), copper powder (0.2 g) and 15 g of K_2CO_3.

*3.3.3.2. 4-Methoxy acridone (**1B**).* The procedure used for **1A** was repeated with 15 g of 2′-methoxydiphenylamine-2-carboxylic acid and 150 g of polyphosphoric acid. The sample of 4-methoxyacridone (**1B**) was recrystallized from methanol/1 N potassium hydroxide (6:2) solution (yield 90%). M.p. 292–293 °C, UV λ_{max} (ε) MeOH) 202 (30 464), 218 (30 861), 256 (102 119) and 400 (11 788) nm; IR: 3409, 3185, 2991, 2818, 1625, 1569, 1528, 1482, 1358, 1334,

1257, 1192, 1081, 968, 821, 756, 695 and 539 cm^{-1}; ^{1}H-NMR: δ 7.17–8.23 (m, Ar–H, 7H, H_1–H_3 and H_5–H_8), 4.05 (s, 3H, OCH_3) and 11.23 (s, 1H, NH); ^{13}C-NMR: δ 176.93 (C_9), 150.21 ($C_{10'}$), 143.20 (C_4), 137.03 ($C_{4'}$), 135.07 (C_6), 127.27 (C_8), 123.71 (C_1), 122.05 ($C_{9'}$), 120.82 ($C_{8'}$), 119.23 (C_3), 117.38 (C_2), 116.16 (C_7), 115.93 (C_5) and 56.15 (OCH_3); MS: *m/z* 226 ($[M + H]^+$, 100). Anal. ($C_{14}H_{11}NO_2$) C, H, N.

*3.3.3.3. 10-(3′-Chloropropyl)-4-methoxyacridone (**2B**).* The procedure used for **2A** was employed with 22.2 mmol of **1B**, 5.9 mmol of tetrabutylammonium bromide and 55.5 mmol of 1-bromo-3-chloropropane. The crude product was purified by column chromatography to give a yellow solid of **2B** (yield 58%). M.p. 82–84 °C, UV λ_{max} (ε) (MeOH) 204 (27 217), 222 (23 208), 260 (75 330) and 395 (11 274) nm; IR: 3445, 2938, 2829, 1716, 1634, 1596, 1480, 1459, 1430, 1369, 1253, 1194, 1068, 962, 818, 754, 694 and 652 cm^{-1}; ^{1}H-NMR: δ 7.30–8.28 (m, Ar–H, 7H, H_1–H_3 and H_5–H_8), 3.93 (s, 3H, OCH_3), 3.68–3.71 (t, 2H, H_m), 3.55–3.58 (t, 2H, H_k) and 2.28–2.32 (m, 2H, H_l); ^{13}C-NMR: δ 176.73 (C_9), 149.73 ($C_{10'}$), 144.26 (C_4), 136.14 ($C_{4'}$), 134.05 (C_6), 133.86 (C_8), 126.39 (C_1), 126.14 ($C_{9'}$), 122.36 ($C_{8'}$), 121.63 (C_3), 118.30 (C_2), 116.94 (C_7), 116.26 (C_5), 56.55 (OCH_3), 49.12 (C_m), 42.81 (C_k) and 31.91 (C_l); MS: *m/z* 303.6 ($(M + 2H)^+$. Anal. ($C_{17}H_{16}ClNO_2$) C, H, N.

*3.3.3.4. 10-(3′-[N-Methylpiperazino]propyl)-4-methoxyacridone (**3B**).* Amounts of 1.13 g (3.7 mmol) of **2B**, 1.5 g of KI, 2.5 g of K_2CO_3 and 0.9 ml (8.1 mmol) of *N*-methylpiperazine were refluxed and processed according to the procedure used for **3A**. The oily product was purified by column chromatography and converted into hydrochloride salt of **3B** (yield 56%). M.p. 230–232 °C, UV λ_{max} (ε) (MeOH) 204 (21 580), 221 (19 270), 258 (69 781) and 396 (10 291) nm; IR: 3432, 2978, 2563, 1614, 1592, 1572, 1464, 1377, 1262, 1204, 1080, 1020, 961, 803, 750 and 694 cm^{-1}; ^{1}H-NMR: δ 7.71–8.69 (m, Ar–H, 7H, H_1–H_3 and H_5–H_8), 3.23 (s, 3H, OCH_3), 2.95 (s, 3H, He), 2.91 (m, 8H, H_a, H_b, H_c and H_d), 2.61–2.65 (m, 4H, H_k and H_n) and 2.28–2.39 (p, 4H, H_l and H_m); ^{13}C-NMR: δ 179.41 (C_9), 149.69 ($C_{10'}$), 144.24 (C_4), 136.04 ($C_{4'}$), 134.12 (C_6), 126.99 (C_8), 123.94 (C_1), 123.79 ($C_{9'}$), 123.39 ($C_{8'}$), 121.39 (C_3), 118.71 (C_2), 117.06 (C_7), 116.84 (C_5), 55.82 (OCH_3), 44.14 (C_k), 42.41 (C_m), 50.01 (C_a and C_b), 49.63 (C_c and C_d), 27.53 (C_e) and 24.88 (C_l); MS: *m/z* 366 $[M + H]^+$.

*3.3.3.5. 10-[3′-N-Piperidinopropyl]-4-methoxyacridone (**4B**).* The experimental steps used for **3B** were repeated by taking 1.08 g (3.58 mmol) of **2B**, 1.5 g of KI, 2.4 g K_2CO_3 and 1.0 ml (10.7 mmol) of piperidine. The crude product was purified to give a pale yellow oily product, which was converted into hydrochloride salt (yield 44%). M.p. 183–185 °C, UV λ_{max} (ε) (MeOH) 203 (18 284), 221 (14 957), 259 (48 048) and 393 (7267) nm; IR: 3509, 3407, 2940, 2636, 2545, 1728, 1591, 1497, 1480, 1430, 1372, 1255, 1165, 1076, 959, 758, 730, 696 and 536 cm^{-1}; ^{1}H-NMR: δ 7.29–8.28 (m, Ar–H, 7H, H_1–H_3 and H_5–H_8), 4.04 (s, 3H, OCH_3), 3.08–3.13 (m, 8H, H_k, H_n, H_a and H_b), 1.68–1.82 (m, 8H, H_l, H_m, H_c and H_d) and 1.30–1.40 (p, 2H, H_e); ^{13}C-NMR: δ 178.42 (C_9), 149.88 ($C_{10'}$), 144.55 (C_4), 136.09 ($C_{4'}$), 134.42 (C_6), 127.11 (C_8), 123.89 (C_1), 123.46 ($C_{9'}$), 123.13 ($C_{8'}$), 121.66 (C_3), 120.56 (C_2), 118.82 (C_7), 117.15 (C_5), 57.23 (OCH_3), 58.89 (C_m), 54.51 (C_k), 50.05 (C_a and C_b), 25.56 (C_l), 24.07 (C_c and C_d) and 22.31 (C_e); MS: *m/z* 351 ($[M + H]^+$, 100). Anal. ($C_{22}H_{26}N_2O_2$) C, H, N.

*3.3.3.6. 10-[3′-N-Morpholinopropyl]-4-methoxyacridone (**5B**).* The procedure used for **4B** was followed with 1.12 g (3.7 mmol) of **2B**, 1.6 g of KI, 2.5 g of K_2CO_3 and 0.84 ml (9.6 mmol) of morpholine. The purified oily product was converted into hydrochloride salt (yield 40%). M.p. 178–179 °C, UV λ_{max} (ε) (MeOH) 203 (18 088), 222 (16 995), 256 (57 617) and 398 (8187) nm; IR: 3460, 2951, 2676, 1654, 1583, 1494, 1428, 1381, 1248, 1193, 1084, 962, 808, 714 and 686 cm^{-1}; ^{1}H-NMR: δ 7.30–8.38 (m, Ar–H, 7H, H_1–H_3 and H_5–H_8), 3.82 (s, 3H, OCH_3), 3.00–3.18 (t, 4H, H_c and H_d), 2.49–2.51 (t, 4H, H_a and H_b), 2.07–2.12 (m, 4H, H_k and H_m) and 0.90–0.94 (t, 2H, H_l); ^{13}C-NMR: δ 176.40 (C_9), 149.30 ($C_{10'}$), 144.31 (C_4), 135.81 ($C_{4'}$), 133.92 (C_6), 126.59 (C_8), 123.30 (C_1), 123.08 ($C_{9'}$), 123.11 ($C_{8'}$), 120.70 (C_3), 118.41 (C_2), 117.31 (C_7), 116.82 (C_5), 62.39 (C_c and C_d), 56.70 (OCH_3), 55.49 (C_a and C_d), 49.13 (C_k), 48.45 (C_m) and 25.31 (C_l); MS: *m/z* 353 ($[M + H]^+$, 100).

*3.3.3.7. 10-(3′-N-[Bis[hydroxyethyl]amino]propyl)-4-methoxyacridone (**6B**).* The method employed for **5B** was used with 1.05 g (3.48 mmol) of **2B**, 1.4 g of KI, 2.4 g of K_2CO_3 and 0.73 ml (7.6 mmol) of diethanolamine. After purification, the yellow oily product was converted into hydrochloride salt (yield 55%). M.p. 212–215 °C, UV λ_{max} (ε) (MeOH) 203 (18 081), 221 (13 605), 259 (42 849) and 393 (6279) nm; IR: 3397, 2966, 2634, 1610, 1595, 1499, 1367, 1261, 1195, 1079, 971, 756 cm^{-1}; ^{1}H-NMR: δ 7.28–8.28 (m, Ar–H, 7H, H_1–H_3 and H_5–H_8), 4.03 (s, 3H, OCH_3), 3.22–3.33 (m, 8H, H_k, H_n, H_a and H_b), 3.79–3.81 (t, 4H, H_c and H_d), 2.51 (s, 2H, H_e and H_f, disappearing on D_2O exchange) and 2.39–2.50 (p, 2H, H_l); ^{13}C-NMR: δ 176.40 (C_9), 149.30 ($C_{10'}$), 144.31 (C_4), 135.81 ($C_{4'}$), 133.92 (C_6), 126.59 (C_8), 123.30 (C_1), 123.08 ($C_{9'}$), 123.11 ($C_{8'}$), 120.70 (C_3), 118.41 (C_2), 117.31 (C_7), 116.82 (C_5), 62.39 (C_c and C_d), 56.70 (OCH_3), 55.49 (C_a and C_d), 49.13 (C_k), 48.45 (C_m) and 25.31 (C_l); MS: *m/z* 371 ($[M + H]^+$, 100). Anal. ($C_{21}H_{26}N_2O_4$) C, H, N.

*3.3.3.8. 10-(3′-N-[(β-Hydroxyethyl)piperazino]propyl)-4-methoxyacridone (**7B**).* Compound **7B** (yield 58%) in the pure form was prepared by following the procedure used for **6B** with 1 g (3.3 mmol) of **2B**, 1.4 g of KI, 2.3 g of K_2CO_3 and 0.97 ml (7.9 mmol) of (β-hydroxyethyl)piperazine. M.p. 189 °C, UV λ_{max} (ε) (MeOH) 203 (35 439), 222 (19 415), 260 (59 708) and 396 (8947) nm; IR: 3395, 2928, 2584, 2373, 1614, 1591, 1557, 1409, 1256, 1205, 1078, 965 and

752 cm^{-1}; ^{1}H-NMR: δ 7.29–8.32 (m, Ar–H, 7H, H_1–H_3 and H_5–H_8), 4.14 (t, 1H, Hg), 4.06 (s, 3H_1, OCH_3), 3.68–3.80 (m, 2H, H_f), 3.07–3.41 (m, 14H, H_k, H_m, H_a, H_b, H_c, H_d and H_e) and 2.50 (t, 2H, H_l); ^{13}C-NMR: δ 176.71 (C_9), 149.67 ($C_{10'}$), 144.11 (C_4), 134.23 ($C_{4'}$), 133.96 (C_6), 126.34 (C_8), 124.60 (C_1), 122.40 ($C_{9'}$), 121.89 ($C_{8'}$), 121.72 (C_3), 118.26 (C_2), 116.96 (C_7), 116.32 (C_5), 57.77 (OCH_3), 57.06 (C_f), 56.33 (C_e), 55.28 (C_k), 49.11 (C_m), 48.20 (C_a and C_b), 47.98 (C_c and C_d) and 24.20 (C_l); MS: *m/z* 396 ([M + H]$^+$, 100).

*3.3.3.9. 10-(4′-Chlorobutyl)-4-methoxyacridone (**8B**).* The experimental steps used for **2B** were followed by taking 5.6 g (24.89 mmol) of **1B**, 2.0 g of (6.9 mmol) of tetrabutylammonium bromide and 62.25 mmol of 1-bromo-4-chlorobutane. The crude product was purified by column chromatography using chloroform/acetone (8:2) to give a bluish solid (yield 52%). UV λ_{max} (ε) (MeOH) 204 (19 173), 222 (14 528), 260 (45 000) and 396 (6850) nm; IR: 3422, 3072, 2958, 1629, 1603, 1496, 1455, 1372, 1254, 1197, 1077, 971, 756 and 682 cm^{-1}; ^{1}H-NMR: δ 7.30–8.28 (m, Ar–H, 7H, H_1–H_3 and H_5–H_8), 4.01 (s, 3H, OCH_3), 3.65–3.68 (t, 2H, H_n), 3.54–3.58 (t, 2H, H_k) and 1.81–1.97 (m, 4H, H_l and H_m); ^{13}C-NMR: δ 176.71 (C_9), 149.67 ($C_{10'}$), 144.11 (C_4), 134.23 ($C_{4'}$), 133.96 (C_6), 126.34 (C_8), 124.60 (C_1), 122.40 ($C_{9'}$), 121.89 ($C_{8'}$), 121.72 (C_3), 118.26 (C_2), 116.96 (C_7), 116.32 (C_5), 57.77 (OCH_3), 57.06 (C_f), 56.33 (C_e), 55.28 (C_k), 49.11 (C_m), 48.20 (C_a and C_b), 47.98 (C_c and C_d) and 24.20 (C_l); MS: *m/z* 317 ([M + H]$^+$, 100%).

*3.3.3.10. 10-[4′-*N*-(Methylpiperazino)butyl]-4-methoxyacridone (**9B**).* Amounts of 1.09 g (3.45 mmol) of **8B**, 1.4 g of KI, 2.3 g of K_2CO_3 and 0.84 ml (7.6 mmol) of *N*-methylpiperazine in acetonitrile were refluxed and worked up according to protocol used for **3B**. The crude oily product was subjected to column chromatography for purification and then converted into hydrochloride salt (yield 57%). M.p. 240–242 °C, UV λ_{max} (ε) (MeOH) 204 (19 834), 221 (17 333), 257 (56 400) and 397 (8267) nm; IR: 3383, 2978, 2618, 1630, 1593, 1487, 1410, 1369, 1245, 1196, 1072, 967, 757 and 676 cm^{-1}; ^{1}H-NMR: δ 7.30–8.28 (m, Ar–H, 7H, H_1–H_3 and H_5–H_8), 4.02 (s, 3H, OCH_3), 2.78–2.85 (m, 8H, H_a, H_b, H_c and H_d), 2.50–2.54 (m, 6H, H_k, H_n and H_e) and 1.04–1.07 (m, 4H, H_l and H_m); ^{13}C-NMR: δ 176.71 (C_9), 149.67 ($C_{10'}$), 144.11 (C_4), 134.23 ($C_{4'}$), 133.96 (C_6), 126.34 (C_8), 124.60 (C_1), 122.40 ($C_{9'}$), 121.89 ($C_{8'}$), 121.72 (C_3), 118.26 (C_2), 116.96 (C_7), 116.32 (C_5), 57.77 (OCH_3), 57.06 (C_f), 56.33 (C_e), 55.28 (C_k), 49.11 (C_m), 48.20 (C_a and C_b), 47.98 (C_c and C_d) and 24.20 (C_l); MS: *m/z* 380 ([M + H]$^+$, 100).

*3.3.3.11. 10-(4′-*N*-Piperidinobutyl)-4-methoxyacridone (**10B**).* The procedure used for **4B** was repeated with 1.0 g (3.2 mmol) of **8B**, 1.38 g of KI, 2.3 g of K_2CO_3 and 0.95 ml (9.6 mmol) of piperidine. The light yellow oil was purified by column chromatography and finally converted into hydrochloride salt (yield 46%). M.p. 182–183 °C, UV λ_{max} (ε) (MeOH) 204 (35 800), 222 (25 437), 261 (84 926) and 397 (12 382) nm; IR: 3403, 2962, 2573, 1632, 1586, 1458, 1403, 1372, 1236, 1184, 1068, 973, 748 and 644 cm^{-1}; ^{1}H-NMR: δ 7.32–8.36 (m, Ar–H, 7H, H_1–H_3 and H_5–H_8), 3.87 (s, 3H, OCH_3), 3.39–3.41 (m, 8H, H_a, H_b, H_k and H_n), 1.69–1.98 (m, 8H, H_c, H_d, H_l and H_m) and 1.21–1.34 (m, 2H, H_e); ^{13}C-NMR: δ 175.79 (C_9), 153.92 ($C_{10'}$), 140.88 (C_4), 136.15 ($C_{4'}$), 133.97 (C_6), 126.73 (C_8), 124.12 (C_1), 122.33 ($C_{9'}$), 120.98 ($C_{8'}$), 120.79 (C_3), 117.96 (C_2), 115.76 (C_7), 106.22 (C_5), 55.64 (C_a and C_b), 55.44 (OCH_3), 52.06 (C_k), 45.02 (C_n), 24.50 (C_l), 22.25 (C_m), 21.56 (C_c and C_d) and 20.41 (C_e); MS: *m/z* 365 ([M + H]$^+$, 87%). Anal. ($C_{23}H_{28}N_2O_2$) C, H, N.

*3.3.3.12. 10-(4′-*N*-Morpholinobutyl)-4-methoxyacridone (**11B**).* Amounts of 1.2 g (3.8 mmol) of **8B**, 1.6 g of KI, 2.6 g of K_2CO_3 and 0.83 ml (9.5 mmol) of morpholine were refluxed and processed according to the procedure used for **10B**. The product was purified by column chromatography and converted to hydrochloride salt (yield 42%). M.p. 223–225 °C, UV λ_{max} (ε) (MeOH) 203 (13 000), 222 (11 311), 260 (38 533) and 396 (5911) nm; IR: 3441, 2939, 2862, 2675, 2620, 1660, 1573, 1500, 1431, 1376, 1259, 1203, 1078, 964, 864, 746 and 488 cm^{-1}; ^{1}H-NMR: δ 7.30–8.28 (m, Ar–H, 7H, H_1–H_3 and H_5–H_8), 4.03 (s, 3H, OCH_3), 3.77–3.97 (m, 4H, H_c and H_d), 3.00–3.14 (m, 8H, H_k, H_n, H_a and H_b), 1.86–1.94 (p, 2H, H_m) and 1.75–1.82 (p, 2H, H_l); ^{13}C-NMR: δ 176.73 (C_9), 149.78 ($C_{10'}$), 144.28 (C_4), 136.23 ($C_{4'}$), 134.12 (C_6), 126.37 (C_8), 124.76 ($C_{9'}$), 122.33 (C_1), 122.09 ($C_{8'}$), 121.62 (C_3), 118.37 (C_2), 117.05 (C_7), 116.45 (C_5), 63.13 (C_c and C_d), 56.92 (C_a and C_b), 55.47 (OCH_3), 51.24 (C_k), 50.85 (C_n), 26.88 (C_l) and 20.36 (C_m); MS: *m/z* 367 ([M + H]$^+$.

*3.3.3.13. 10-(4′-*N*-[Bis[hydroxyethyl]amino]butyl)-4-methoxyacridone (**12B**).* The protocol used for **6B** was repeated with 1.13 g (3.58 mmol) of **8B**, 1.5 g of KI, 2.5 g of K_2CO_3 and 0.73 ml (7.6 mmol) of diethanolamine. The bluish thick liquid was purified by column chromatography and then converted into hydrochloride salt (yield 58%). M.p. 202–203 °C, UV λ_{max} (ε) (MeOH) 204 (48 118), 221 (36 353), 259 (28 588) and 397 (16 235) nm; IR: 3386, 2932, 1630, 1601, 1483, 1432, 1374, 1253, 1198, 1071, 958, 876, 820, 757, 696 and 544 cm^{-1}; ^{1}H-NMR: δ 7.30–8.28 (m, Ar–H, 7H, H_1–H_3 and H_5–H_8), 4.03 (s, 3H, OCH_3), 3.76–3.78 (t, 4H, H_c and H_d), 3.21–3.30 (m, 8H, H_k, H_n, H_a and H_b), 2.51 (s, 2H, H_e and H_f, disappearing on D_2O exchange), 1.85–1.95 (p, 2H, H_m) and 1.72–1.81 (p, 2H, H_l); ^{13}C-NMR: δ 176.73 (C_9), 149.77 ($C_{10'}$), 144.28 (C_4), 136.23 ($C_{4'}$), 134.11 (C_6), 126.33 (C_8), 124.72 (C_1), 122.30 ($C_{9'}$), 122.00 ($C_{8'}$), 121.60 (C_3), 118.33 (C_2), 117.06 (C_7), 116.43 (C_5), 79.22 (C_c and C_d), 56.86 (C_a and C_b), 55.33 (OCH_3), 52.90 (C_k), 51.26 (C_n), 26.73 (C_l) and 20.50 (C_m); MS: *m/z* 385 ([M + H]$^+$, 12). Anal. ($C_{22}H_{28}N_2O_4$) C, H, N.

*3.3.3.14. 10-(4′-*N*-[(β-Hydroxyethyl)piperazino]butyl)-4-methoxyacridone (**13B**).* Compound **13B** (yield 50%) as hydrochloride salt was prepared according to the procedure

used for **7B** with 1.2 g (3.8 mmol) of **8B**, 1.5 g of KI, 2.5 g of K_2CO_3 and 1.07 ml (8.7 mmol) of (β-hydroxyethyl)piperazine. It was purified by column chromatography. M.p. 142–143 °C, UV λ_{max} (ε) (MeOH) 203 (35 564), 222 (23 064), 260 (52 097) and 396 (7500) nm; IR: 3313, 2976, 2446, 1625, 1597, 1497, 1430, 1372, 1255, 1201, 1073, 963, 816, 750, 693 and 543 cm^{-1}; ^{1}H-NMR: δ 7.30–8.27 (m, 7H, Ar–H, H_1–H_3 and H_5–H_8), 4.54 (t, 1H, H_g), 4.02 (s, 3H, OCH_3), 3.64–3.77 (m, 2H, H_f), 3.02–3.60 (m, 14H, H_a, H_b, H_c, H_d, H_e, H_k and H_n) and 1.00–1.10 (m, 4H, H_l and H_m); ^{13}C-NMR: δ 176.40 (C_9), 149.80 ($C_{10'}$), 144.30 (C_4), 134.12 ($C_{4'}$), 134.10 (C_6), 126.35 (C_8), 124.77 (C_1), 122.34 ($C_{9'}$), 121.63 ($C_{8'}$), 118.36 (C_3), 117.08 (C_2), 116.48 (C_7), 116.40 (C_5), 56.91 (–OCH_3), 55.28 (C_f), 51.23 (C_e), 48.04 (C_k), 40.16 (C_n), 38.92 (C_c and C_d), 30.67 (C_a and C_b), 26.76 (C_l) and 20.82 (C_m); MS: *m/z* 410 ($[M + H]^+$, 100).

4. Biological activity

4.1. Cell lines and cell culture

4.1.1. GC_3/c1 cells

A cloned line of human colon adenocarcinoma which is intrinsically resistant to vincristine (approximately fourfold relative to KB-3-1), was routinely grown at 37 °C in antibiotic-free RPMI-1640 medium supplemented with 2 mM glutamine and 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air.

4.1.2. KB-3-1 and KBCh^R-8-5 cells

Human KB epidermoid carcinoma cells were subcloned two times; a single cloned 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 vinblastine (~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^{-1} of colchicine. KBCh^R-8-5 and GC_3/c1 cells overexpressed *mdr1* and were positive for Pgp as determined by immunocytochemistry using HYB241 and C219 monoclonal antibodies.

4.1.3. Accumulation studies

Two milliliters of cell suspension (2×10^6 cells) were plated in 35 × 10 mm style "easy grip" culture dishes (Greiner GmbH, Germany) and allowed to attach to plastic overnight at 37 °C. Medium was aspirated and cells were washed with (2 × 2 ml) 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 min in PT buffer prior to aspiration and adding 1 cm^3 of serum-free RPMI-1640 HEPES buffer (10.4 g RPMI-1640 medium in 1 l of 25 mM HEPES, pH 7.4) containing 49.9 nM [^{3}H]vinblastine (0.5 μCi ml^{-1}, specific activity 9.4 Ci $mmol^{-1}$; Amersham Pharmacia Biotech) with or without verapamil (Sigma) or acridone derivatives (**1A–13A** or **1B–13B**) at 100 μM dissolved in DMSO (final culture concentration < 0.1% DMSO). After 2 h 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^{-1}: 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 min 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 vinblastine was determined (Table 1).

4.1.4. Measurement of vinblastine efflux

Cells (2×10^6 per 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 min in another 2 ml of the same buffer. KBCh^R-8-5 cells were incubated with 1 ml of RPMI-1640 (serum free) HEPES buffer, pH 7.4 containing 37.43 nM [^{3}H] vinblastine for 2 h at room temperature. Drug solutions were aspirated and 1 ml of the same buffer without or with modulator (**1A–13A** or **1B–13B**) or verapamil at 100 μM were added and incubated for 2 h more 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.

4.1.5. Inhibition of cellular growth

KB-3-1, KBCh^R-8-5 and GC_3/c1 at 5000 cells per well were plated in triplicate in six-well flat bottom tissue culture plates (Greiner GmbH). After 24 h at 37 °C in an atmosphere of 5% CO_2–95% air, medium was replaced with 3 ml of fresh medium containing acridone compounds (**1A–13A** or **1B–13B**) at concentration ranging from 0 to 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 2 ml of 0.9% saline and dried overnight. 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.

4.1.6. Effect of N^{10}-substituted acridones on in vitro cytotoxicity of vinblastine

To determine the effects of the modulators on the cytotoxicity, cells were treated with serial dilutions of vinblasine (upto 100 nM for KBCh^R-8-5 or GC_3/c1 and upto 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 vinblastine in the absence of modulator by the IC_{50} in the presence of modulating agent.

5. Isolation and purification of calmodulin from bovine testes

The protein has been isolated and purified according to the method described by Chafouleas et al. [31]. Briefly, approximately 1 kg of bovine testes was homogenized and centrifuged at 20 000 × *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 × *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 h. The dialyzed protein solution was applied to a DEAE–cellulose column (1.5 × 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 h^{-1} flow rate. Fractions (5 cm^3) 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 ultramembrane and the sample applied to a Sephadex G-100 column (1.5 × 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.

5.1. 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 microprocessor system controller, two model 110A single-piston reciprocating pumps, a dynamically stirred gradient mixing chamber, a model 210 sample injection valve, μBondapak ODS, 5 μ reverse phase steel column (4.6 × 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 ($R = 0.998$).

5.2. 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+}, 1.25×10^{-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 (1 U 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.

5.3. Inhibition of calmodulin dependent cAMP-phosphodiesterase activity by acridones (**1A–13A** or **1B–13B**)

Optimal assay conditions remain same as above. The assays were carried out in the presence and absence of different concentrations of acridone derivatives, 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-phosphodiesterase 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.

6. Statistical analysis

Statistical analysis of the data was performed to calculate *P*-values using Graphpad Instat 3.0 program.

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