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Acridones circumvent P-glycoprotein-associated multidrug resistance (MDR) in cancer cells

Vadiraj S. Gopinath,[a,*] Padma Thimmaiah[b] and Kuntebommanahalli N. Thimmaiah[b]

[a]*Advinus Therapeutic Pvt. Ltd, 21 & 22, Phase-II, Peenya Industrial Area, Bangalore 560 058, India*
[b]*Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN 38104, USA*

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Abstract—Multidrug resistance (MDR) mediated by overexpression of *MDR1* P-glycoprotein (P-gp) is one of the best characterized transporter-mediated barriers to successful chemotherapy in cancer patients. Chemosensitizers are the agents that increase the sensitivity of multidrug-resistant cells to the toxic influence of previously less effective drugs. In an attempt to find such vital chemosensitizers, a series of N^{10}-substituted-2-chloroacridone analogous (**1**–**17**) have been synthesized. Compound **1** was prepared by the Ullmann condensation of *o*-chlorobenzoic acid and *p*-chloroaniline followed by cyclization. The *N*-(ω-chloroalkyl) analogues were found to undergo iodide catalyzed nucleophilic substitution reaction with secondary amines and the resultant products were characterized by spectral methods. The lipophilicity expressed in $\log_{10}P$ and pK_a of compounds has been determined. All compounds were examined for their ability to increase the uptake of vinblastine (VLB) in MDR $KBCh^R$-8-5 cells and the results showed that the compounds **6**, **8**, **11**–**14**, **16**, and **17** at their respective IC_{50} concentrations caused a 1.0- to 1.7-fold greater accumulation of VLB than did a similar concentration of the standard modulator, verapamil (VRP). Results of the efflux experiment showed that VRP and each of the modulators significantly inhibited the efflux of VLB, suggesting that they may be competitors for P-gp. All modulators effectively competing 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 and the results showed that modulators **11**, **13**, **14**, **16**, and **17** were able to completely reverse the 25-fold resistance of $KBCh^R$-8-5 cells to VLB. Examination of the relationship between lipophilicity and antagonism of MDR showed a reasonable correlation suggesting that hydrophobicity is one of the determinants of potency for anti-MDR activity of 2-chloroacridones. The results allowed us to draw preliminary conclusions about structural features of 2-chloroacridones important for MDR modulation.

1. Introduction

Resistance to chemotherapy represents a major obstacle in the treatment of cancer. Many tumors are intrinsically resistant to chemotherapy, whereas others initially respond to treatment, but acquire resistance to selected cytotoxic drugs during chemotherapy. Multidrug resistance (MDR) can be broadly defined as a phenomenon by which cultured cells in vitro and tumor cells in vivo show resistance simultaneously to a variety of structurally and functionally dissimilar cytotoxic and xenobiotic compounds.[1–6] While several different genes have been shown to be associated with a multidrug resistance phenotype,[7–9] MDR mediated by overexpression of the *MDR1* gene product, P-gp, represents one of the best characterized barriers to chemotherapeutic treatment in cancer.[6,10] P-gp has been shown to bind ATP and drug analogues,[11,12] has ATPase activity,[13] and catalyzes ATP-dependent drug efflux to effectively reduce intracellular accumulation in resistant cells.[14] Because clinical studies have documented the poor outcomes associated with *MDR1* P-gp expression in tumors,[15,16] reversal of MDR by nontoxic agents that block the transport activity of *MDR1* Pgp has been an important target for pharmaceutical development. Regardless of mode of interaction, the net effect is that Pgp reduces the intracellular concentration of substrates in Pgp-expressing multidrug-resistant cells compared with non-P-gp-expressing cells. The mechanism of reversal of this resistance is believed to be competition between the modulator and cytotoxic drug for binding to the ATP-dependent efflux pump, P-gp.

Because of the importance of MDR in clinical oncology, an intensive search for the new MDR reversal agents is

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* Corresponding author. Tel.: +91 80 28394959; fax: +91 80 28394015; e-mail: vadiraj.gopinath@advinus.com

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still an unceasing challenge for the scientists since the demonstration that verapamil has this property.[17] Consequently, a large number of small molecules capable of modulating P-gp mediated MDR have been described in the literature. When co-administered with a cytotoxic agent, these nontoxic modulators enhance net accumulation of relevant cytotoxic drugs within the tumor cells. Many compounds known to have other pharmacological sites of action initially were used to reverse MDR in cancer cells grown in culture and several underwent pilot clinical trials.[18] These compounds included verapamil, cyclosporine A, quinidine, trifluoperazine, and their derivatives.[18] However, these agents had limited clinical utility because of unacceptable toxicities at serum levels of drug needed to modulate MDR1 Pgp.[15] Second generation modulators, such as dexverapamil, an optically pure verapamil,[19] and PSC 833 (Valspodar), a cyclic undecapeptide analogue of cyclosporine A,[20] were soon developed with improved efficacy. These were followed by third generation modulators, such as GF 120918, a substituted isoquinolinyl acridonecarboxamide,[21] LY335979, a difluorocyclopropyl dibenzosuberane,[22] VX710 (biricodar), an amido-ketopipecolinate,[23] XR9576 (tariquidar), an analogue of anthranilamide pharmacophore,[24–26] and MS209, a quinoline-derivatized analogue,[27,28] that have been developed more recently. Thus, the MDR phenotype may be modulated more effectively with these more selective reversal agents to improve the efficacy of chemotherapeutic agents by delivering an appropriate dose at the targeted site. However, unfortunately, most of these agents suffer clinically from their intrinsic toxicity or from undesired effects on the pharmacokinetics of the accompanying anticancer drugs. These limitations have spurred on efforts to search for new and more effective compounds. Therefore, the development of potent P-gp inhibitors that are better tolerated remains an important task in successfully overcoming multidrug resistance. In the course of an on-going program aimed at identifying potent MDR modulators, the investigators' group has already 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.[29] Subsequently, 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 results revealed that a chloro-group at C-2 position exhibited the highest anti-MDR activity as compared to methoxy substituent at C-2 or C-4 position.[30,31] Therefore, to extend the portfolio of better transporter antagonists, the authors describe the synthesis and biological evaluation of a series of 17 2-chloro-N^{10}-substituted acridone derivatives as potential MDR reversing agents for use in cancer chemotherapy.

2. Chemistry

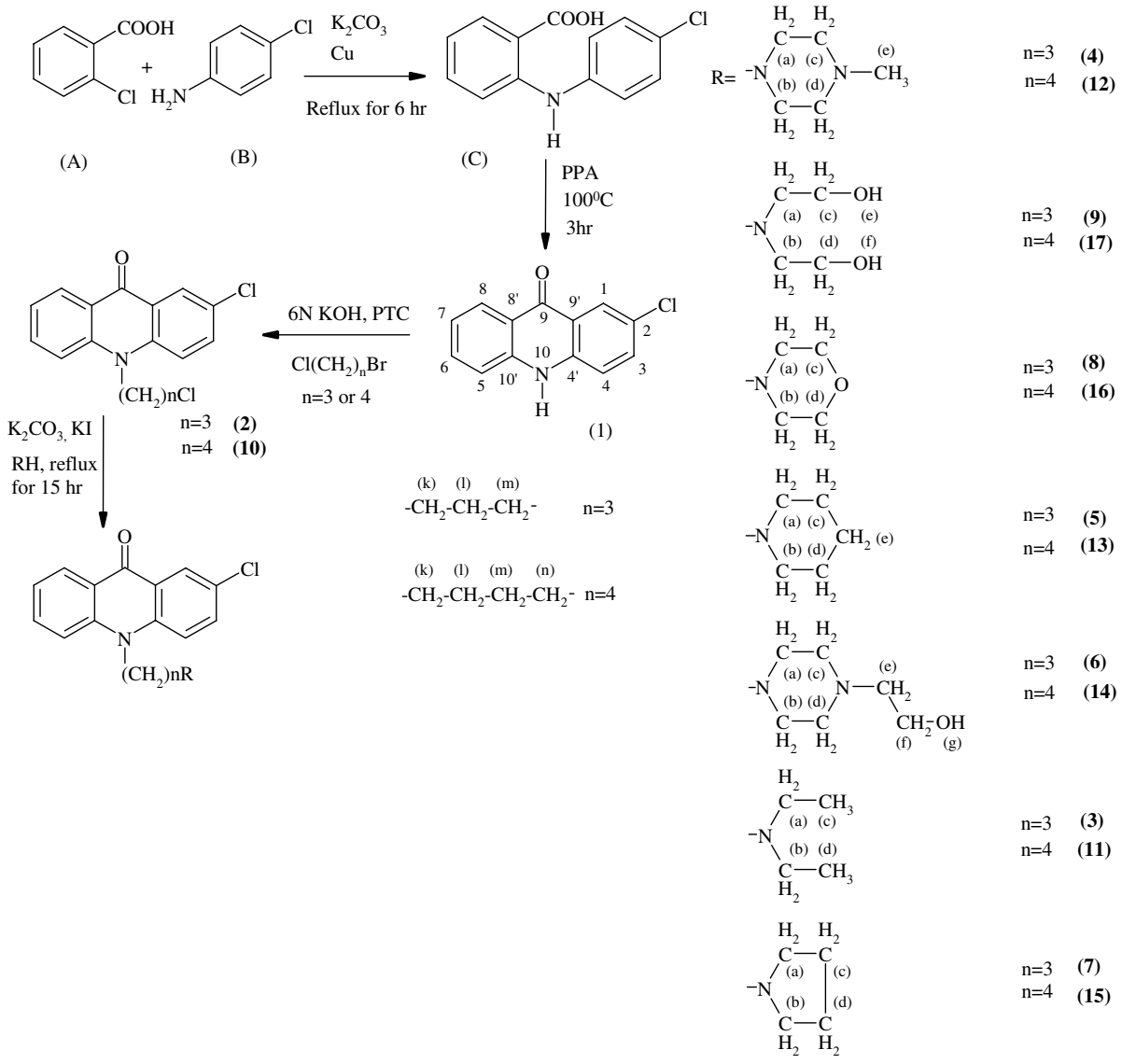
Easy synthesis, attractive coloration, and crystallinity of acridine compounds have attracted the attention of the chemists.[32] The basic tricyclic framework can be decorated with suitable substituents to confer specificity against both prokaryotic and eukaryotic targets which have given acridines a respectable reputation in the history of chemotherapy.[33] 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.[33–37] In the present study, 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 acridone derivatives.

The chemical synthesis of 2-chloro-N^{10}-substituted acridone derivatives has been carried out according to Scheme 1. Parent 2-chloroacridone (**1**) was prepared by the Ullmann condensation of *o*-chlorobenzoic acid and 4-chloroaniline to form 2-(4-chloro-phenylamino) benzoic acid. The reactants were heated preferably at reflux in the presence of copper powder and K_2CO_3 in isoamyl alcohol medium. The 2-(4-chloro-phenylamino) benzoic acid was cyclized with sulfuric acid at 100 °C on a water-bath to form 2-chloroacridone (**1**) (~60%) and sulfonated 2-chloroacridone (~40%) as evidenced by TLC. When cyclization was carried out with polyphosphoric acid instead of sulfuric acid on a water-bath at 100 °C only a single product of 2-chloroacridone (**1**) with better yield (~90%) was obtained.

The weakly basic nature of nitrogen atom of the acridone nucleus usually resists to undergo N-alkylation with alkyl halides. However, it can be achieved in the presence of basic condensing agents like sodium amide or sodium hydride. The general procedure for preparing *N*-alkyl derivative consists of condensation of acridone with requisite alkyl halide in the presence of a strong acid binding agent like sodium amide in anhydrous solvents such as toluene or benzene. The reaction of 2-chloroacridone with mixed chlorobromoalkanes in the presence of sodium amide in anhydrous toluene under reflux conditions gave N^{10}-(chloroalkyl)-2-chloroacridone, but the yield was low, besides requiring drastic experimental conditions. To overcome this drawback, N^{10}-alkylation was carried out in the presence of a phase transfer catalyst (PTC), which is easier to work with and gives better yield than the previously described method.

Stirring of 2-chloroacridone (**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 10-(3′-chloropropyl) (**2**) or 10-(4′-chlorobutyl) (**10**) acridones in good yield. Here, ammonium salt transports hydroxide ion from aqueous phase to organic phase where the actual reaction takes place. These results are interpreted by deprotonation of the acridone by the OH^-, transferred by the catalyst into the organic layer. The anion formed may be regarded as phenolate stabilized anion, which subsequently undergoes alkylation to form the aromatized system.

Iodide catalyzed nucleophilic substitution of the N^{10}-chloropropyl or N^{10}-chlorobutyl acridone with



Scheme 1.

various secondary amines [*N*,*N*-diethylamine, *N*,*N*-diethanolamine, pyrrolidine, piperidine, morpholine, 1-methylpiperazine and (β-hydroxyethyl)piperazine] by refluxing for different times in the presence of K_2CO_3 in acetonitrile, gave the free bases **3**–**9** and **11**–**17**. All 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, ^1H and ^{13}C NMR, and mass spectral analyses.

The UV-spectral data of acridone derivatives are in close agreement with the spectral characteristics of analogous heterocyclic compounds.[38–40]

The IR spectra revealed the characteristic absorption of aromatic ketone and that of a secondary amino group for parent compound or that of a tertiary amino group for the derivatives. The strong bands in the region 1728–1612 cm^{-1} were assigned to >C=O stretching of the aromatic ring system. The >C=O stretching frequency was lower than the expected value in most of the compounds because the conjugation was brought up by two aromatic rings and chloro-group that enhanced the mesomeric shift. Thus, the characteristic bands in the IR spectra support the structure of acridones.

The ^1H NMR spectrum of 2-chloroacridone showed seven aromatic protons at 'δ' 7.2–8.34 (m, H_1, H_3, H_4–H_8) for propyl and butyl derivatives. A singlet at 'δ' 12.06 was assigned to N–H proton of the aromatic ring. Also this signal was not showed up in rest of the compounds indicating that '–H' atom is replaced by an alkyl side chain. A common singlet at 'δ' 2.51 was exhibited by two protons (H_e and H_f) in all bishydroxyethylamino substituted acridones and disappeared on D_2O exchange. Similarly, signals at 'δ' 4.53 due to –OH group was commonly found in all (β-hydroxyethyl)piperazino acridones, which disappeared on D_2O exchange. Comparison of the ^1H NMR spectral data of parent 2-chloroacridone with those of corresponding N^{10}-substituted acridones showed that chemical shift of all the

common hydrogen atoms slightly shifted downfield (~0.2 ppm). The strong interactions between neighboring protons (spin–spin coupling) which exist between *ortho*-hydrogens ($J \sim 5$–8 Hz) and weaker couplings by *meta*-hydrogens ($J \sim 1$–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 the case of all compounds is fully supported by the integration curves and all derivatives showed the characteristic chemical shifts for the acridone nucleus.

The ^{13}C NMR spectrum of each of N^{10}-substituted-2-chloroacridones showed 13 signals representing 13 magnetically different environmental aromatic carbons. Signal appeared at 'δ' 176.62 in the spectrum of **1** 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 the deshielding effect offered by carbonyl oxygen. The signals at 'δ' 121.75 and 120.87 ppm were assigned to the bridged head carbons $C_{9'}$ and $C_{8'}$, respectively. The chemical shift at 'δ' 127.91 and 133.81 ppm were assigned to the *ortho*-carbons (C_1 and C_8) and at 'δ' 126.01 and 135.95 ppm to the *para*-carbons (C_3 and C_6). Similar explanation for assigning the carbons holds good for rest of the compounds. The assignments of the ^{13}C-resonances of all 2-chloroacridone derivatives are in close agreement with an analogues compound N^{10}-alkyl substituted 9(10*H*)-acridone. In toto, ^1H and ^{13}C NMR spectral data were consistent with the proposed structures.

The mass spectra of the compounds were analyzed by using electrospray ionization technique and the spectral features are similar. The preponderance of $[M+2H]^+$ in the ESI spectra of 2-chloroacridone is a testimony to the stability of the acridone ring nucleus. Molecular ions were observed in the form of $[M+2H]^+$ in the spectra of these acridone derivatives. In general, mass spectral features of these compounds are similar and straightforward. 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.

3. Pharmacological results and discussion

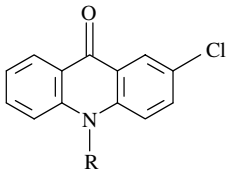
3.1. pK_a and lipophilicity effects

The effectiveness of an MDR reversal agent will depend in part on its ability to accumulate in cells. The 2-chloroacridones are weak bases that exist in both charged (protonated) and uncharged (unprotonated) forms. The unprotonated or neutral form of compounds would be highly membrane permeable and be 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 be 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 8.29 to 9.55 lie closer to physiological pH, which may suggest that these compounds accumulate in MDR cells as free bases rather than in protonated form. As hydrophobicity was often reported to be an important feature of MDR modulators, we decided to determine $\log_{10}P$ values for acridones studied. The lipophilicity data varying from 1.38 to 2.25 for 17 acridone derivatives (**1**–**17**) are given in Table 1. Within the series, all compounds are highly lipophilic at pH 7.4 and it is expected that they will accumulate rapidly into the cells. Of note was that the $\log_{10}P$ values of butyl substituted acridones were comparatively higher when compared to propyl substituted counterparts. Additionally, it is speculated that the acridone nucleus with chlorine at position C-2 may exhibit higher affinity for membranes or be more readily taken up into cells than that which is unsubstituted. Furthermore, analysis of the relationship between lipid solubility and the effectiveness of the chemosensitizers to increase VLB accumulation in drug-resistant KBCh^R-8-5 cells showed a good correlation (Fig. 1; $R^2 = 0.50$). The major outlier in this analysis was that the degree of lipophilicity of each drug would seem to be important and it is one of the determinants of potency for the P-gp modulating activity of 2-chloroacridones.

3.2. Effect of 2-chloro-N^{10}-substituted acridones on the uptake of VLB in KBCh^R-8-5 cells

The effect of 2-chloro-N^{10}-substituted acridones on the uptake of VLB was determined in MDR KBCh^R-8-5 cells. As shown in Table 1, compounds (**1**–**17**) at their IC_{50} concentrations (from Table 3) exhibited significant VLB accumulation enhancing effect (7- to 20-fold relative to control) compared to a standard modulator verapamil (VRP) (11.9-fold). Nine compounds **1**–**5**, **7**, **9**, **10**, and **15** were found to possess less VLB enhancing effect (7.0- to 10.0-fold) compared to VRP (11.9-fold) relative to control. Remaining eight compounds (**6**, **8**, **11**–**14**, **16**, and **17**) caused a 1.0- to 1.7-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 a very little effect in sensitive KB-3-1 cells (data not shown). Phenoxazines were shown to exhibit a similar effect on the uptake of VLB in drug-sensitive KB-3-1 cells.[41] For propyl and butyl series, the VLB uptake enhancing effects with respect to control are in the range 798–1372% and 982–2010%, respectively, suggesting that the butyl derivatives seem to rank the list. Further, the results of the experiments using variable concentrations of modulators revealed that all the modulators at 5, 10, and 50 μM 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 (data not shown). The greatest increase was for derivative **14** and this was probably due to the enhanced

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



Compound	R	pK_a	$\log_{10}P$[a]	VLB uptake (%control)[b] ± SEM
1	H	8.29	2.06	715 ± 2.30
2	$(CH_2)_3$—Cl	8.65	1.38	880 ± 1.70
3	$(CH_2)_3$—N(C_2H_5)(C_2H_5)	5.37 9.28	1.54	948 ± 2.50
4	$(CH_2)_3$—N(piperazine)—N—CH_3	5.28 9.15	1.48	1010 ± 2.40
5	$(CH_2)_3$—N(piperidine)	5.39 9.45	1.52	927 ± 2.70
6	$(CH_2)_3$—N(piperazine)—N—CH_2CH_2OH	5.26 9.21	1.76	1252 ± 3.30
7	$(CH_2)_3$—N(pyrrolidine)	5.21 9.32	1.55	988 ± 3.50
8	$(CH_2)_3$—N(morpholine)—O	4.87 9.20	1.64	1372 ± 2.80
9	$(CH_2)_3$—N(CH_2CH_2OH)(CH_2CH_2OH)	4.80 9.30	1.47	1017 ± 2.90
10	$(CH_2)_4$—Cl	8.85	1.68	982 ± 1.60
11	$(CH_2)_4$—N(C_2H_5)(C_2H_5)	5.45 9.31	1.90	1356 ± 1.90
12	$(CH_2)_4$—N(piperazine)—N—CH_3	5.61 9.30	1.95	1420 ± 1.70
13	$(CH_2)_4$—N(piperidine)	5.36 9.55	2.01	1855 ± 2.40
14	$(CH_2)_4$—N(piperazine)—N—CH_2CH_2OH	5.26 9.21	2.25	2010 ± 3.60
15	$(CH_2)_4$—N(pyrrolidine)	5.28 9.50	1.74	1010 ± 3.20
16	$(CH_2)_4$—N(morpholine)—O	4.91 9.20	1.89	1690 ± 1.90

Table 1 (*continued*)

Compound	R	pK_a	$\log_{10}P$[a]	VLB uptake (%control)[b] ± SEM
17	$(CH_2)_4$—N(CH_2CH_2OH)(CH_2CH_2OH)	4.80 9.30	1.67	1219 ± 2.60
Verapamil		ND	ND	1190 ± 3.80

ND, not determined because there was no inflection between pH 2 and 12.
Compounds were tested at their respective IC_{50} concentration (Table 3). All values represent the mean of two separate experiments and each experiment was done in triplicate.
[a] Octanol/buffer partition coefficient.
[b] $\left(\frac{\text{VLB uptake with modulator}}{\text{VLB uptake without modulator}}\right) \times 100$.

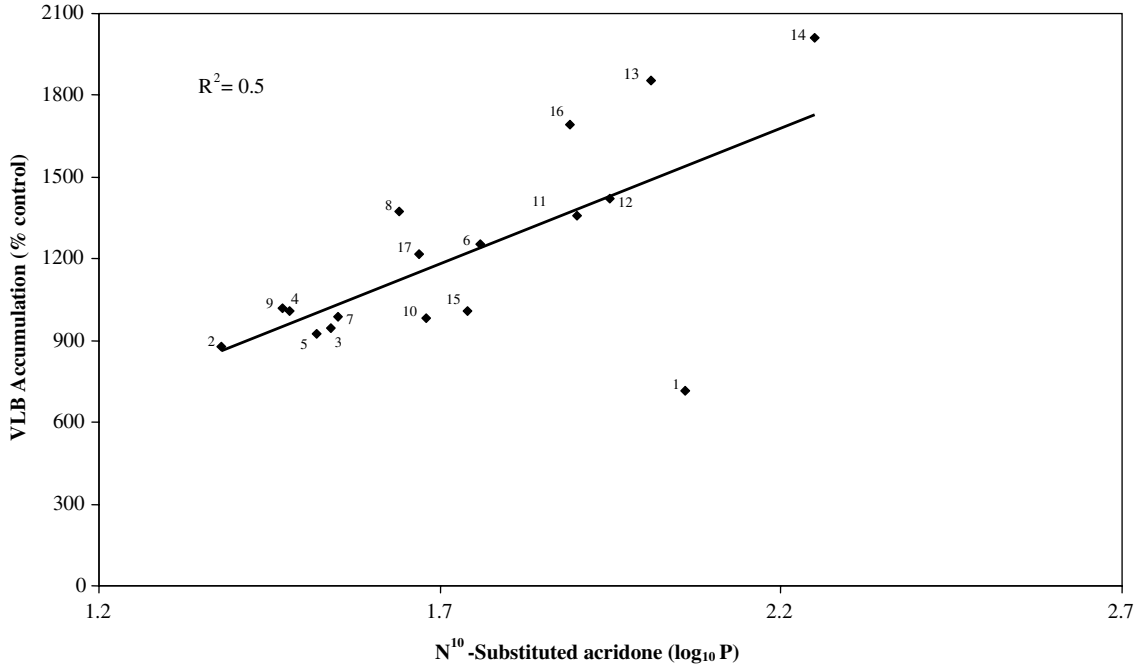


Figure 1. Correlation between the $\log_{10}P$ of the 2-chloroacridones (**1**–**17**) and their ability to enhance vinblastine accumulation in $KBCh^R$-8-5 cells.

lipophilicity of the compounds after –Cl substitution. Additionally, it is speculated that the acridone nucleus with –Cl 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 vinblastine uptake suggesting that N^{10}-substitution is required for optimal activity. Comparison of the derivatives for their 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 derivatives, although there are exceptions in the series.

3.3. Effect of substituted acridones on the efflux of [^{3}H]VLB from $KBCh^R$-8-5 cells

In the efflux experiments, the cell associated radiolabel remaining after 2 h 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 and each of the modulators at their IC_{50} values 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 h of incubation in the absence of modulator. Out of 17 compounds tested, only eight modulators exhibited greater efflux inhibiting activity than VRP. Furthermore, the time-course of VLB efflux from MDR cells was performed (Fig. 2), for which the cells were loaded with 49.9 nM [^{3}H]VLB for 2 h at room temperature and washed and the retained radioactivity was measured at the indicated times. In the absence of modulator, less than 20% of the cellular VLB remained after 2 h. When a similar experiment in the presence of 50 μM VRP or 4.5 μM (IC_{50}) of **14** was performed, about 67% or 80% of the cell-associated VLB remained at the end of the efflux period. These data suggest that the 2-chloroacridones, like VRP, are able to inhibit P-gp mediated VLB efflux from $KBCh^R$-8-5 cells.

3.4. 2-Chloroacridones interact with P-gp by photolabeling this protein with [^{3}H]azidopine

P-gp has been suggested to play a key role in the development of MDR and one piece of experimental data that directly connects reduced drug accumulation and

Table 2. Effect of 2-chloro-N^{10}-substituted acridones on the cellular retention of VLB in KBCh^{R}-8-5 cells after 2 h efflux

Compound	Cellular retention (%) of VLB after 2 h efflux
1	49.00
2	40.00
3	47.00
4	51.00
5	54.50
6	56.50
7	56.00
8	62.00
9	66.00
10	60.50
11	77.20
12	75.00
13	77.00
14	80.50
15	79.00
16	76.00
17	74.00
Verapamil	67.00
Control	20.00

All values represent the mean of two separate experiments with a SD of less than 8% of the mean; each experiment was done in triplicate. Modulators were used at their respective IC_{50} concentration (Table 3).

active transport of a variety of agents is found in the ability of several photoactivable compounds to covalently bind to P-gp. Most commonly used photolabeling agents which seem to predominantly label P-gp are VLB derivative, *N*-(*p*-azido-[3-^{125}I]salicyl)-*N*′-β-aminoethylvindesine (NASV) and a dihydropyridine derivative, azidopine. Cornwell et al.[42] originally showed that P-gp of the MDR KB cells is specifically labeled with VLB analogues and that verapamil blocked the specific labeling. A dihydropyridine analogue, azidopine, has been found to specifically interact with P-gp by a photolabeling assay. The reversing of drug resistance by chemosenstizers seems to be correlated with the inhibition of [^{3}H]azidopine photolabeling of P-gp. Photolabeling was not detected under our experimental conditions when membranes prepared from drug-sensitive KB-3-1 cells or from KBCh^{R}-8-5 cells, which have a low level of drug resistance (data not shown), were used. A highly VLB resistant and P-gp-expressing KB cell subline KB-VI was therefore used for [^{3}H]azidopine competition studies.

The VLB transport and cell survival data suggest that the modulatory effect of 2-chloroacridone derivatives is through an interaction with P-gp. To confirm this, the competition between [^{3}H]azidopine and 17 2-chloroacridone derivatives was determined. At their IC_{50} concentrations, 2-chloroacridones inhibited the labeling of P-gp by [^{3}H]azidopine. The binding of [^{3}H]azidopine to P-gp after inhibition by acridones expressed in percentage of control (no competitor) is as follows: **1** by 85%, **2** by 83%, **3** by 80%, **4** by 45%, **5** by 50%, **6** by 35%, **7** by 50%, **8** by 30%, **9** by 40%, **10** by 70%, **11** by 20%, **12** by 20%, **13** by 15%, **14** by 10%, **15** by 30%, **16** by 15%, **17** by 20%, and VRP by 65%. Comparison of the data on the competition between [^{3}H]azidopine and each acridone modulator at its IC_{50} revealed that the ability of all the compounds, except compounds **1**–**3** and **10**, 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. Since 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. 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-chloroacridones may be mediated through P-gp dependent mechanism.

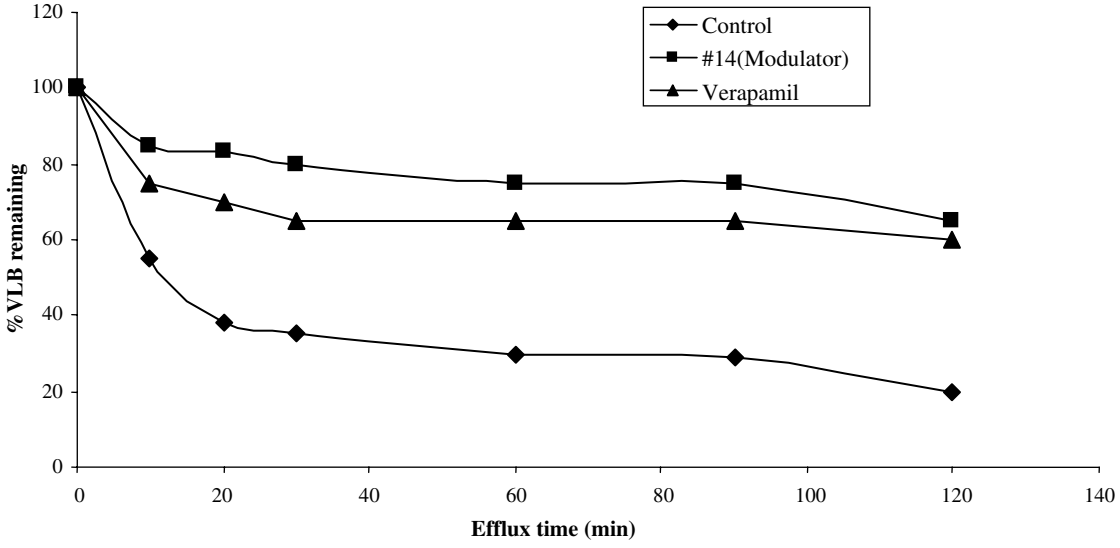


Figure 2. Effect of modulator **14** and verapamil on the outward transport of [^{3}H]vinblastine from KBCh^{R}-8-5 cells. Efflux studies were done in the presence of vehicle control, 0.1% DMSO, modulator **14** or verapamil as described in Section 4.

3.5. In vitro cytotoxicity of acridones

The cytotoxicity of 17 compounds (**1**–**17**) was examined in KBCh^R-8-5 by incubating cells with several concentrations (0.001, 0.01, 1.0, 2.5, 5.0, 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 all the compounds lie, respectively, in the range 0.46–1.41 μM and 4.5–18.0 μM for KBCh^R-8-5 cells. Careful examination of IC_{50} values for N^{10}-chloropropyl (7.5–15.5 μM) and N^{10}-chlorobutyl derivatives (4.5–12.50 μM) against KBCh^R-8-5 cells revealed that antiproliferative activity largely increased as the chain length increased from 3 to 4 suggesting that the hydrophobicity plays an important role in the biological activity. Increasing the distance between the ring nucleus and amino group increased the antiproliferative activity of these compounds. Therefore, from this study we can tentatively conclude that the structural features required within the series to cause a maximum antiproliferative 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. Further, modulators (**11**, **13**, **14**, **16**, and **17**) which completely reversed the drug resistance in KBCh^R-8-5 cells, were examined for cytotoxicity against the drug-sensitive (KB-3-1) cells. In the case of sensitive cells, the IC_{10} values (data not given) and the respective IC_{50} values of 3.9, 4.2, 1.5, 3.5, and 4.5 μM for **11**, **13**, **14**, **16**, and **17** were obtained. The results demonstrate that KB-3-1 cells are more sensitive to acridones compared to KBCh^R-8-5 cells.

3.6. Sensitization of MDR cells by acridone derivatives

We have evaluated all the 17 compounds for their ability 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 cells is summarized in Table 4. The modulators tested at their IC_{10} enhanced the cytotoxicity of VLB by 3.7- to 32-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**–**17**) lie in the range of 2.1–19.1 nM. Examination of IC_{50} values of VLB in the presence of propyl derivatives (3.60–16.1 nM) or butyl derivatives (2.1–9.2 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 modulators revealed that the modulators (**11**, **13**, **14**, **16**, and **17**) demonstrated the greatest effect. Only five acridones, 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 72 nM in KBCh^R-8-5 cells in the absence of modulating agent. The most effective modulators (**11**, **13**, **14**, **16**, and **17**) in KBCh^R-8-5 cells were subsequently tested in KB-3-1 and all were shown to cause a small sensitization (2.0- to 5.0-fold) of this drug-sensitive line to VLB. However, a similar degree of sensitization was also found when the classical MDR modulator, VRP (4-fold), was used. Of note was that the acridones are most effective in reversing MDR cells than drug-sensitive KB-3-1 cells.

Table 3. Cytotoxicity of acridone derivatives in human MDR cells

Compound	KBCh^R-8-5 cells		Compound	KBCh^R-8-5 cells	
	IC_{10} (μM)	IC_{50}[a] (μM)		IC_{10} (μM)	IC_{50}[a] (μM)
1	1.41	18.00	**10**	1.25	10.8
2	1.35	15.00	**11**	0.62	9.10
3	1.00	12.50	**12**	0.80	8.00
4	0.80	9.00	**13**	0.60	5.20
5	1.25	15.5	**14**	0.46	4.50
6	0.74	7.50	**15**	1.85	12.50
7	0.90	14.0	**16**	0.72	6.00
8	1.15	10.50	**17**	0.60	5.00
9	1.20	8.70	—	—	—

IC_{10} and IC_{50} are the concentrations (μM) required to produce 10% and 50% reduction, respectively, in clonogenic survival of cells under the conditions described in Section 4.

[a] Each experiment was done in triplicate.

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 P-gp mediated MDR include a tricyclic hydrophobic acridone ring with a –Cl group at position C-2 and a diethylamino-, piperidino-, bishydroxyethylamino, 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 VLB accumulation are among the compounds that can completely reverse VLB resistance in KBCh^R-8-5 cells. Further, examination of the relationship between hydrophobicity and VLB accumulation in KBCh^R-8-5 cells showed a reasonable correlation. Thus, the degree of lipophilicity of each drug, although important, was not the sole determinant of potency for anti-MDR activity.

Summing up, in the present work new effective MDR modulators were identified among 2-chloroacridone derivatives. Of particular note is that from among 17 modulators investigated, only five of them were able to reverse completely the 25-fold resistance of KBCh^R-8-5 cells to VLB and therefore, these five compounds constitute the promising anti-MDR agents that should be further tested in other model systems.

Table 4. Effect of acridone modulators on potentiation of vinblastine cytotoxicity in drug-resistant KBCh^R-8-5 cells

Compound[a]	KBCh^R-8-5 cells		Compound[a]	KBCh^R-8-5 cells	
	Vinblastine IC_{50}[b] (nM)	Fold-potentiation		Vinblastine IC_{50}[b] (nM)	Fold-potentiation
1	19.1	3.60	**10**	9.20	7.5
2	16.1	4.28	**11**	2.30	Complete[c]
3	13.1	5.30	**12**	4.10	16.8
4	9.40	7.34	**13**	2.25	Complete[c]
5	13.0	5.30	**14**	2.30	Complete[c]
6	4.50	15.3	**15**	9.10	7.58
7	8.40	8.20	**16**	2.10	Complete[c]
8	3.60	19.2	**17**	2.45	Complete[c]
9	5.90	11.6	—	—	—

[a] Modulator used at the IC_{10} concentration (Table 3).
[b] Concentration of modulators required for 50% reduction of cells compared to controls.
[c] Complete reversal of VLB resistance.

4. Experimental

4.1. General

Reactions were monitored by thin layer chromatography. For column chromatography silica gel (Merck, grade 60–120, 230–400 mesh, 60 Å) was used. Melting points were recorded on an Electoral-9100 melting-point apparatus and are uncorrected. UV-spectra were recorded in methanol on a UV-160A SHIMADZU spectrophotometer. Infrared spectra as KBr pellets in the range 4000–400 cm^{-1} were obtained on a Bio-Rad model 165 Fourier transform spectrophotometer. ^{1}H-(400 MHz) and ^{13}C-(400 MHz) spectra were recorded in DMSO-(d_6) or D_2O solution in a 5-mm tube on a Bruker amx 400 and drx 500 Fourier transform spectrometer with tetramethylsilane (TMS) as internal standard. The spectrometer was internally locked to the deuterium frequency of the solvent. Chemical shifts (δ) were recorded in ppm relative to TMS. Acridone derivatives were analyzed by electron ionization (EI) technique using an Autospec Q (VG Analytical, Manchester, UK) hybrid tandem mass spectrometer of E_1BE_2-qQ geometry. Only the front end (i.e., E_1BE_2) was used for this study. The data accumulation and manipulation were under digital Vax Station 3100-based Opus software. The following experimental conditions were used. Electron ionization–ionizing energy, 70 eV; emission current, 100 μA; source temperature, 100 °C; mass resolution 2000; accelerating potential, 8000 V; mass range 10–500 Da; and spectral scan rate, 5 s/decade; samples were introduced via direct insertion probe, which was not heated; the only source of heat to the sample was through contact with the ion source.

4.2. Materials

All chemicals and supplies were obtained from standard commercial sources unless otherwise indicated. Vinblastine sulfate was purchased from Cetus Corporation (Emeryville, CA, USA). RPMI-1640 medium with glutamine and without sodium bicarbonate and sodium pyruvate was purchased from Gibco-BRL (Grand Island, NY, USA). DMEM medium, Hank's balanced salt, and trypsin–EDTA were purchased from Imperial (UK). [^{3}H]Vinblastine (Sp. Act. 9.4 Ci $mmol^{-1}$) was purchased from Amersham Pharmacia Biotech., UK Ltd. Verapamil hydrochloride, colchicines, and dimethyl sulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

4.3. Synthesis

4.3.1. Preparation of 4′-chlorodiphenylamine-2-carboxylic acid.

4.3.1.1. Ullmann condensation. To a mixture of *o*-chlorobenzoic acid (10 g, 0.064 mol), *p*-chloroaniline (8.1 g, 0.064 mol), and copper powder (0.2 g) in 60 mL of isoamylalcohol, dry K_2CO_3 (10 g) was slowly added and the contents were refluxed for 6 h. The isoamylalcohol was removed by steam distillation and the mixture poured into 1 L of hot water, and acidified. 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, light yellowish precipitate was obtained which was washed with hot water and recrystallized from aqueous methanol to give a light yellow solid, 4′-chlorodiphenylamine-2-carboxylic acid (yield 13.4 g, 84%, mp 186 °C).

4.3.1.2. Cyclization of 4′-chlorodiphenylamine-2-carboxylic acid to 2-chloroacridone (1). 4′-Chlorodiphenylamine-2-carboxylic acid (10 g,) was taken in a flask to which was added 100 g of polyphosphoric acid. The reaction mixture was heated on a water-bath at 100 °C for 3 h with stirring. 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. The yellow precipitate that formed was filtered, washed with hot water and collected. The sample of 9 (10-H)-2-chloroacridone (**1**) was recrystallized from acetic acid (yield 7.41 g, 80%, mp 398 °C). UV λ_{max} (ε) (MeOH): 214 (23,135), 257 (43,240), 299 (2616), 386 (7482) nm. IR: 3655, 2987, 2855, 1627, 1164, 960, 754, 683 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 7.18–8.21 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), and 12.06 (s, N–H). ^{13}C NMR (DMSO-d_6): δ 127.92(C_1), 113.1(C_2), 126.00(C_3), 120.09(C_4), 117.63(C_5), 135.95(C_6), 121.2(C_7), 133.82(C_8), 175.64(C_9), 113.08($C_{9'}$), 139.77($C_{4'}$), 140.79($C_{10'}$) and 121.75($C_{8'}$). MS: *m/z* (%) 231 [$(M+H)^+$, 100]. Anal. ($C_{13}H_8NOCl$) C, H, N.

4.3.2. Synthesis of N^{10}-alkylated 2-chloroacridones via phase transfer catalysis.

4.3.2.1. 10-(3′-Chloropropyl)-2-chloroacridone (2). 2-Chloroacridone (6 g, 0.026 mol) was dissolved in tetrahydrofuran (100 mL) and then 6 N aqueous potassium hydroxide (50 mL) and tetrabutylammonium bromide (2 g, 0.006 mol) were added to it. Stirred at room temperature for 30 min and added 1-bromo-3-chloropropane (0.065 mol) slowly into the reaction mixture and stirred for an additional 48 h 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 chromatography to give a yellow solid of **2** (yield 6.2 g, 52%, mp 141 °C). UV λ_{max} (ε) (MeOH): 214 (22,819), 254 (36,633), 299 (2491), 399 (6732) nm. IR: 2940, 1628, 1460, 1044, 961, 753, 682 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.36–8.38 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 3.78–3.81 (t, 2H, H_k), 3.85–3.91 (t, 2H, H_m), and 2.32–2.5 (m, 2H, H_l). ^{13}C NMR (DMSO-d_6): 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($C_{9'}$), 139.99($C_{4'}$), 141.01($C_{10'}$), 116.97($C_{8'}$), 44.08(C_k), 30.19(C_l) and 41.85(C_m). MS: *m/z* (%) 308 [$(M+H)^+$, 100]. Anal. ($C_{16}H_{13}NOCl_2$) C, H, N.

4.3.2.2. 10-(3′-*N*-Diethylaminopropyl)-2-chloroacridone (3). To the solution of **2** (1.12 g, 3.66 mmol) in 60 mL of acetonitrile was added 1.57 g KI and 2.54 g K_2CO_3 and stirred at reflux conditions for 30 min. Then diethylamine (1.17 g, 16.02 mmol) was added slowly. The reaction mixture was refluxed for 18 h, cooled to room temperature, and extracted with chloroform. The chloroform layer was washed with water thrice, dried over anhydrous sodium sulfate, and rotavaporated. The product was purified by column chromatography to give a yellow oily product which was converted into hydrochloride salt of **3** (yield 0.55 g, 40%, mp 110–112 °C). UV λ_{max} (ε) (MeOH): 218 (15,250), 255 (27,850), 391 (5200), 410 (5800) nm. IR: 3504, 2919, 1724, 1591, 1263, 948, 753, 673, 544 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.35–8.34 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 3.09–3.81 (m, 8H, H_k, H_m, H_a, H_b), 2.08–2.5 (t, 2H, H_e) and 1.23–1.27 (m, 6H, H_c and H_d). ^{13}C NMR (DMSO-d_6): δ 128.38(C_1), 121.89(C_2), 126.64(C_3), 116.01(C_4), 113.74(C_5), 136.61(C_6), 121.87(C_7), 134.82(C_8), 175.74(C_9), 22.96($C_{9'}$), 140.84($C_{4'}$), 141.22($C_{10'}$), 118.80($C_{8'}$), 58.87(C_k), 22.94(C_l), 24.42(C_m), 51.61(C_a and C_b) and 13.33(C_c and C_d). MS: *m/z* (%) 346 [$(M+H)^+$, 100]. Anal. ($C_{20}H_{24}N_2OCl_2$) C, H, N.

4.3.2.3. 10-[3′-*N*-(Methylpiperazino)propyl]-2-chloroacridone (4). The experimental procedure used for **3** is applicable with 1.25 g (4.08 mmol) of **2**, 1.76 g of KI, 2.86 g of K_2CO_3 and 1.38 g (13.7 mmol) of *N*-methylpiperazine. The oily residue was purified by column chromatography and converted into hydrochloride salt of **4** (yield 0.8 g, 42%, mp 268 °C). UV λ_{max} (ε) (MeOH): 217 (19,993), 256 (58,980), 394 (15,245), 412 (16,744) nm. IR: 3399, 2958, 1610, 1494, 1267, 1058, 958, 755, 685 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.26–8.34 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 2.08–3.77 (m, 12H, H_k, H_m, H_a, H_b, H_c and H_d), 2.5 (s, 3H, H_e) and 1.87–2.27 (m, 2H, H_l). ^{13}C NMR (DMSO-d_6): 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($C_{9'}$), 139.34($C_{4'}$), 141.36($C_{10'}$), 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* (%) 371 [$(M+H)^+$, 100].

4.3.2.4. 10-(3′-*N*-Piperidinopropyl)-2-chloroacridone (5). The procedure used for **3** was repeated with 1.2 g (3.92 mmol) of **2**, 1.75 g of KI, 2.74 g of K_2CO_3, 1.25 g (14.82 mmol) of piperidine. The purified product was converted into hydrochloride salt (yield 0.75 g, 49%, mp 246–250 °C). UV λ_{max} (ε) (MeOH): 216 (24,357), 255 (37,928), 382 (5964), 400 (6904) nm. IR: 3384, 2982, 1625, 1465, 958, 754, 663 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.34–8.33 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 3.31–3.41 (m, 8H, H_k, H_m, H_a, H_b), 2.25–2.89 (m, 2H_l, H_c and H_d), and 1.69–1.84 (m, 3H, H_e). ^{13}C NMR (DMSO-d_6): 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($C_{9'}$), 139.90($C_{4'}$), 141.15($C_{10'}$), 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* (%) 356 [$(M+H)^+$, 100]. Anal. ($C_{21}H_{24}N_2OCl_2$) C, H, N.

4.3.2.5. 10-(3′-*N*-[(β-Hydroxyethyl)piperazino]propyl)-2-chloroacridone (6). The method employed for **3** was used with 1.0 g (3.26 mmol) of **2**, 1.41 g of KI, 2.28 g of K_2CO_3 and 2.06 g (15.8 mmol, 1.94 mL) of (β-hydroxyethyl)piperazine. The oily residue was purified by column chromatography and it was converted into hydrochloride salt of **6** (yield 0.62 g, 40%, mp 260–262 °C). UV λ_{max} (ε) (MeOH): 216 (18,600), 255 (54,068), 386 (8851), 404 (10,310) nm. IR: 3368, 2958, 1611, 1560, 1459, 1270, 961, 758, 685 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.15–8.34 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 4.55 (s, –OH), 3.26–3.6 (m, 12H, H_e, H_m, H_a, H_b, H_c, H_d), 3.82 (m, 2H, H_k), 4.46 (m, 2H, H_f) and 1.38–1.4 (m, 4H, H_l, H_m). ^{13}C NMR (DMSO-d_6): 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($C_{9'}$), 140.22($C_{4'}$), 141.26($C_{10'}$), 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* (%) 401 [$(M+H)^+$, 100]. Anal. ($C_{22}H_{28}N_3O_2Cl_3$) C, H, N.

4.3.2.6. 10-[3′-*N*-Pyrrolidinopropyl]-2-chloroacridone (7). Amounts of 1.02 g (3.33 mmol) of **2**, 1.65 g of KI, 2.64 g of K_2CO_3, and 0.88 g (0.8 mL, 8.34 mmol) of piperidine were refluxed and processed according to the procedure used for **3**. The crude product was purified by column chromatography and the pale yellow oily product was converted into hydrochloride salt of **7** (yield 0.65 g, 48%, mp 183–185 °C). UV λ_{max} (ε) (MeOH): 217 (15,324), 256 (31,465), 386 (5183), 405 (5972) nm. IR: 3393, 2947, 1621, 1492, 1457, 1267, 961, 758, 682 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.38–8.40 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 3.14–3.94 (m, 8H, H_k, H_m, H_a, H_b), and 1.56–2.5 (m, 2H_l, H_c and H_d). ^{13}C NMR (DMSO-d_6): 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($C_{9'}$), 140.24($C_{4'}$), 141.18($C_{10'}$), 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* (%) 342 [$(M+H)^+$, 100].

4.3.2.7. 10-(3′-*N*-Morpholinopropyl)-2-chloroacridone (8). Compound **8** as its hydrochloride salt (yield 0.6 g, 43%, mp 248–250 °C) was obtained by following the procedure of **3** with 1.1 g of **2** (3.59 mmol), 1.55 g KI, 2.5 g of K_2CO_3, and 1.17 g (13.4 mmol) of morpholine. UV λ_{max} (ε) (MeOH): 217 (23,445), 256 (54,741), 389 (7075), 408 (11,260) nm. IR: 3429, 2869, 1617, 1494, 1272, 874, 684 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.36–8.35 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 3.04–3.10 (t, 4H, H_c and H_d), 2.27 (m, 4H, H_k, H_m), 2.50 (t, 4H, H_a, H_b), and 1.21–1.97 (m, 2H, H_l). ^{13}C NMR (DMSO-d_6): 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), 176.34(C_9), 126.52($C_{9'}$), 139.72($C_{4'}$), 141.00($C_{10'}$), 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* (%) 358 [$(M+H)^+$, 100]. Anal. ($C_{20}H_{22}N_2O_2Cl_2$) C, H, N.

4.3.2.8. 10-(3′-*N*-[Bis[hydroxyethyl]amino]propyl)-2-chloroacridone (9). The experimental steps used for **3** were repeated with 1 g (3.26 mmol) of **2**, 1.48 g of KI, 2.3 g of K_2CO_3, and 0.88 g (8.34 mmol) of *N*,*N*-diethanolamine. The crude product was purified by column chromatography to give a light yellow solid **9** (yield 0.65 g, 48%, mp 148–150 °C). UV λ_{max} (ε) (MeOH): 216 (30,000), 255 (61,447), 386 (9659), 402 (10,681) nm. IR: 3286, 2973, 2885, 1630, 1488, 1267, 960, 757, 682 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.35–8.34 (m, Ar-H, 7H, H_1 H_3, H_4 and H_5–H_8), 3.25–3.28 (t, 4H, H_k, H_m), 3.53–3.79 (t, 8H, H_a, H_b), 3.81–3.92 (m, 4H, H_c and H_d), 2.5 (s, 2H, H_e and H_f, disappearing on D_2O exchange), and 2.07–2.08 (q, 2H, H_l). ^{13}C NMR (DMSO-d_6): δ 128.38(C_1), 122.55(C_2), 126.65(C_3), 116.04(C_4), 113.83(C_5), 136.50(C_6), 121.23(C_7), 134.69(C_8), 175.36(C_9), 121.88($C_{9'}$), 140.32($C_{4'}$), 141.22($C_{10'}$), 118.80($C_{8'}$), 47.36(C_k), 21.09(C_l), 42.78(C_m), 46.22(C_a and C_b) and 8.52(C_c and C_d). MS: *m/z* (%) 376 [$(M+H)^+$, 100]. Anal. ($C_{20}H_{23}N_2O_3Cl$) C, H, N.

4.3.2.9. 10-(4′-Chlorobutyl)-2-chloroacridone (10). Yellow crystals of compound **10** in the pure form (yield 6.5 g, 55%, mp 101–106 °C) were prepared by following the procedure used for **2** with 6 g (0.026 mol) of 2-chloroacridone and 1-bromo-4-chlorobutane (0.065 mmol). UV λ_{max} (ε) (MeOH): 217 (15,914), 254 (31,930), 392 (8004), 412 (8687) nm. IR: 3395, 2928, 1614, 1591, 1256, 965, 752 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.25–8.3 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 3.61–3.73 (t, 4H, H_k, H_m), and 1.86–3.34 (m, 4H, H_l, H_m). ^{13}C NMR (DMSO-d_6): δ 126.75(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($C_{9'}$), 140.03($C_{4'}$), 141.29($C_{10'}$), 117.67($C_{8'}$), 44.70(C_k), 25.51(C_l), 29.26(C_m) and 44.96(C_n). MS: *m/z* (%) 320 [$(M+H)^+$, 100]. Anal. ($C_{17}H_{15}NOCl_2$) C, H, N.

4.3.2.10. 10-(4′-*N*-Diethylaminobutyl)-2-chloroacridone (11). The procedure used for **3** was followed with 1.2 g (3.8 mmol) of **10**, 1.57 g of KI, 2.64 g of K_2CO_3, and 1.3 g (17.8 mmol) of *N*,*N*-diethylamine. The product was purified by column chromatography to give a yellow oily product which was converted into its hydrochloride form of **11** (yield 0.73 g, 50%, mp 100–104 °C). UV λ_{max} (ε) (MeOH): 216 (23,266), 255 (36,367), 392 (6864) nm. IR: 3386, 2941, 1625, 1458, 1276, 960, 756 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.35–8.35 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 4.48–4.52 (t, 2H, H_k), 3.08–3.81 (m, 8H, H_m, H_a, H_b), 1.22–1.26 (m, 6H, H_c and H_d) and 1.83–2.5 (t, 4H, H_l, H_n). ^{13}C NMR (DMSO-d_6): 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($C_{9'}$), 140.13($C_{4'}$), 141.37($C_{10'}$), 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* (%) 358 [$(M+H)^+$, 100]. Anal. ($C_{21}H_{26}N_2OCl_2$) C, H, N.

4.3.2.11. 10-(4′-*N*-(Methylpiperazino)butyl)-2-chloroacridone (12). Amounts of 1.1 g of **10** (3.43 mmol), 1.42 g of KI, 2.37 g of K_2CO_3, and 1.56 g (15.6 mmol) of *N*-methylpiperazine were refluxed and processed according to the procedure used for **11**. The crude product was chromatographed on silica gel to get the pure base which was then converted into hydrochloride salt of 10-(4′-*N*-(methylpiperazino)butyl)-2-chloroacridone **12** (yield 0.8 g, 57%, mp 260–262 °C). UV λ_{max} (ε) (MeOH): 216 (18,164), 256 (59,249), 392 (16,542), 412 (18,380) nm. IR: 3445, 2829, 1716, 1634, 1480, 1253, 962, 754, 652 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.34–8.32 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 2.8–3.7 (m, 12H, H_k, H_m, H_a, H_b, H_c, H_d), 2.5 (s, 3H, H_e) and 1.84–2.5 (m, 4H, H_l, H_m). ^{13}C NMR (DMSO-d_6): 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($C_{9'}$), 140.06($C_{4'}$), 141.31($C_{10'}$), 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 and C_d) and 42.02(C_e). MS: *m/z* (%) 385 [$(M+H)^+$, 100].

4.3.2.12. 10-(4′-*N*-Piperidinobutyl)-2-chloroacridone (13). Compound **10** (1.5 g, 4.68 mmol), KI (1.94 g), K_2CO_3 (3.23 g), and piperidine (1.4 g, 23.42 mmol) were used for this reaction and the rest of the steps used for **12** remains the same. The oily residue was then converted into hydrochloride salt of **13** (yield 1 g, 70%, mp 199–200 °C). UV λ_{max} (ε) (MeOH): 217 (15,900), 259 (36,500), 393 (8367), 411 (9400) nm. IR: 3400, 2880, 1629, 1595, 1459, 1263, 959, 758, 682 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.36–8.36 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 3.31–3.41 (m, 8H, H_k, H_m), 1.38–1.40 (m, 8H, H_a, H_b, H_c and H_d). 1.68–1.96 (m, 2H, H_e) and 2.81–2.84 (q, 4H, H_n, H_l). ^{13}C NMR (DMSO-d_6): 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($C_{9'}$), 140.14($C_{4'}$), 141.37($C_{10'}$), 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* (%) 370 [$(M+H)^+$, 100]. Anal. ($C_{22}H_{26}N_2OCl_2$) C, H, N.

4.3.2.13. 10-(4′-*N*-[(β-Hydroxyethyl)piperazino]butyl)-2-chloroacridone (14). The procedure used for **6** was repeated with 1.1 g (3.43 mmol) of **10**, 1.42 g of KI, 2.37 g of K_2CO_3, and 1.56 g (14.6 mmol, 1.5 mL) of (β-hydroxyethyl)piperazine. The oily residue was purified by column chromatography and dissolved in anhydrous acetone and treated with ethereal hydrochloride to give hydrochloride salt of **14** (yield 0.62 g, 40%, mp 260–262 °C). UV λ_{max} (ε) (MeOH): 216 (25,436), 260 (29,499), 393 (5526), 410 (6203) nm. IR: 3509, 2940, 1728, 1480, 1255, 959, 758, 696 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.31–8.3 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 4.64 (s, 1H, H_g), 2.49–3.26 (m, 12H, H_e, H_m, H_a, H_b, H_c, H_d), 3.82 (m, 2H, H_k), 4.46 (m, 2H, H_f) and 1.38–1.4 (m, 4H, H_l, H_m). ^{13}C NMR (DMSO-d_6): 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($C_{9'}$), 140.03($C_{4'}$), 141.28($C_{10'}$), 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) and 55.33(C_e). MS: *m/z* (%) 413 [$(M+H)^+$, 100]. Anal. ($C_{23}H_{30}N_3O_2Cl_3$) C, H, N.

4.3.2.14. 10-[4′-*N*-Pyrrolidinobutyl]-2-chloroacridone (15). The procedure employed for **7** was used with 0.85 g (2.65 mmol) of **10**, 1.1 g of KI, 1.85 g of K_2CO_3, and 0.942 g (1.1 mL, 13.25 mmol) of pyrrolidine. The crude product was purified by column chromatography to give a pale yellow oily product, which was then converted into hydrochloride salt of 10-[4′-*N*-pyrrolidinobutyl]-2-chloroacridone **15** (yield 0.8 g, 57%, mp 268–271 °C). UV λ_{max} (ε) (MeOH): 205 (10,784), 222 (16,995), 256 (57,617), 398 (8187) nm. IR: 3460, 2951, 1654, 1428, 1248, 962, 686 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.36–8.36 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 3.31–3.41 (m, 8H, H_k, H_m), 1.38–1.40 (m, 8H, H_a, H_b, H_c and H_d) and 2.81–2.84 (q, 4H, H_n, H_l). ^{13}C NMR (DMSO-d_6): 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($C_{9'}$), 140.07($C_{4'}$), 141.32($C_{10'}$), 121.51($C_{8'}$), 53.61(C_k), 17.56(C_l), 22.83(C_m), 45.15(C_n), 50.41(C_a and C_b) and 24.20(C_c and C_d). MS: *m/z* (%) 370 [$(M+H)^+$, 100]. Anal. ($C_{21}H_{24}N_2OCl_2$) C, H, N.

4.3.2.15. 10-(4′-*N*-Morpholinobutyl)-2-chloroacridone (16). Repeated the procedure used for **8** with 0.9 g (2.81 mmol) of **10**, 1.16 g of KI, 2.5 g of K_2CO_3, and 0.98 g (11.24 mmol) of morpholine to get an oily product, which was purified by column chromatography. Light colored oil thus obtained was converted into hydrochloride salt of 10-(4′-*N*-morpholinobutyl)-2-chloroacridone **16** (yield 0.65 g, 56%, mp 238–240 °C). UV λ_{max} (ε) (MeOH): 203 (18,088), 222 (16,995), 256 (57,617), 398 (8187) nm. IR: 3397, 2966, 1610, 1261, 971, 756 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.3–8.34 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 3.14–3.10 (t, 4H, H_c and H_d), 2.37 (m, 4H, H_k, H_m), 2.50 (t, 4H, H_a, H_b), and 1.29–1.91 (m, 2H, H_l). ^{13}C NMR (DMSO-d_6): 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($C_{9'}$), 140.06($C_{4'}$), 141.31($C_{10'}$), 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* (%) 372 [$(M+H)^+$, 100].

4.3.2.16. 10-(4′-*N*-[Bis[hydroxyethyl]amino]butyl)-2-chloroacridone (17). The procedure used for **9** was followed with 1 g (3.12 mmol) of **10**, 1.3 g of KI, 2.2 g of K_2CO_3, and 1 g (9.58 mmol) of diethanolamine. The product was purified by column chromatography to give a pale yellow solid **17** (yield 0.7 g, 53%, mp 243 °C). UV λ_{max} (ε) (MeOH): 217 (8626), 255 (24,792), 392 (6942), 412 (7650) nm. IR: 3422, 2958, 1629, 1496, 1455, 1254, 971, 756, 682 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.34–8.34 (m, Ar-H, 7H, H_1, H_3, H_4 and H_5–H_8), 3.40–3.49 (t, 4H, H_k, H_m), 2.08–2.57 (m, 4H, H_l, H_a, H_b), 1.65–1.9 (m, 4H_l, H_c and H_d) and 2.5 (s, 2H, H_e and H_f, disappearing on D_2O exchange). ^{13}C NMR (DMSO-d_6): 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($C_{9'}$), 140.13($C_{4'}$), 141.39($C_{10'}$), 121.55($C_{8'}$), 59.33(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* (%) 390 [$(M+H)^+$, 100]. Anal. ($C_{21}H_{25}N_2O_3Cl$) C, H, N.

4.4. Biological activity

4.4.1. Determination of pK_a and partition coefficient. The pK_as were determined according to our previously published method.[30] The relative lipophilicity at pH 6.8 was assessed for each of the derivative using an adaptation of the method of Zamora et al.[43] This method involves measuring the partitioning of the compound between 1-octanol and 0.1 M PBS buffer (pH 7.4). The partition coefficient, *P*, was determined by dividing the concentration of the derivative in 1-octanol by the concentration in the aqueous phase.

4.4.2. Cell lines and cell culture. 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 VLB (10-fold). KB-3-1, $KBCh^R$-8-5, and KB-V1 cells were grown in monolayer culture in antibiotic-free Dulbecco's modified Eagle's 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 and KB-V1 cells was maintained by culturing them, respectively, with 10 ng/mL of colchicine and 1 ng/mL of VLB. $KBCh^R$-8-5 and KB-V1 cells overexpressed *mdr 1* and were positive for P-gp as determined by immunocytochemistry using HYB241 and C219 monoclonal antibodies.

4.4.3. Accumulation studies. Two milliliters of cell suspension (2×10^6 cells) was 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 mL 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]VLB (0.5 μCi mL^{-1}, specific activity 9.4 Ci mmol^{-1}; Amersham Pharmacia Biotech) with or without VRP or 2-chloro-N^{10}-substituted acridones (**1**–**17**) at their respective IC_{50} concentrations (Table 3), 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 a 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 (Table 1).

4.4.4. Measurement of VLB efflux. Cells (2×10^6/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 min in another 2 mL of the same buffer. KBCh^R-8-5 cells were incubated with 1 mL of serum-free RPMI-1640 Hepes buffer, pH 7.4, containing 49.9 nM [^{3}H]VLB for 2 h at room temperature. Drug solutions were aspirated and 1 mL of the same buffer without or with modulators (**1**–**17**) at their IC_{50} concentrations or VRP (50 μM) was added and incubated for an additional 2 h 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 and the results are given in Table 2.

4.4.5. Competition for [^{3}H]azidopine labeling of P-gp

4.4.5.1. Preparation of plasma membrane fractions and photoaffinity labeling. Competition assay for photolabeling of P-gp used membranes from KB-V1cells, 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.[44] For photoaffinity labeling, membrane protein (200 mg) 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 mmol^{-1}; Amersham) in the absence or presence of modulators at their IC_{50} values, in a total volume of 150 mL. After incubating for 20 min in the dark, the mixture was then irradiated with a germicidal UV light (30 W), commonly used in laminar flow hoods, for 20 min at a distance of 10 cm.

4.4.5.2. SDS–PAGE and autoradiography. Photolabeled proteins were separated by one-dimensional 5–15% SDS–PAGE under reducing conditions using the discontinuous buffer system of Laemmli.[45] After staining with Coomassie Blue and destaining, the gels were soaked in Amplify (Amersham Corp.) for 30 min and dried under vacuum at 75 °C. The dried gels were exposed to film for 2–3 days at 37 °C and developed. Radioactively labeled bands were scanned in their centers with a densitometer and quantitated by integration with a recorder.

4.4.6. Inhibition of cellular proliferation. Five thousand cells (KB-3-1or KBCh^R-8-5) per well were plated in triplicate in 6-well flat-bottom tissue culture plates (Greiner GmbH, Germany). After incubating for 24 h in a CO_2 (5%) incubator, medium was replaced with 2 mL of fresh medium containing acridones (**1**–**17**) at concentration ranging from 0 to 100 μM in DMSO (final culture concentration <0.1%, DMSO) and cells were incubated at 37 °C for further 7 days. The medium was aspirated and cells were washed once with 2 mL of 0.9% saline and dried overnight. Colonies were stained with 1 mL of 0.1% crystal violet followed by washing twice with distilled water and dried overnight. The IC_{10} and IC_{50} values (Table 3) 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.

4.4.7. Effect of 2-chloro-N^{10}-substituted acridones on in vitro cytotoxicity of VLB. To determine the effects of the modulators on the cytotoxicity of VLB, KBCh^R-8-5 cells were treated with serial dilutions of VLB (up to 100 nM) 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 (Table 4) was calculated by dividing the IC_{50} for VLB in the absence of modulator by the IC_{50} in the presence of modulating agent.

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