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Synthesis and evaluation of acridine- and acridone-based anti-herpes agents with topoisomerase activity

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Abstract—The discovery of new non-nucleoside antiviral compounds is of significant and growing interest for treating herpes virus infections due to the emergence of nucleoside-resistant strains. Using a whole cell virus-induced cytopathogenic assay, we tested a series of substituted triaryl heterocyclic compounds including acridones, xanthones, and acridines. The compounds which showed activity against Herpes Simplex-1 and/or Herpes Simplex-2 were further assayed for inhibition of topoisomerase activity to gain insight into the mechanism of action. The results indicate that the acridine analogs bearing substituted carboxamides and bulky 9-amino functionalities are able to inhibit herpes infections as well as inhibit topoisomerase II relaxation of supercoiled DNA. Given the mechanism of action of amsacrine (a closely related, well-studied 9-amino substituted acridine), the compounds were further tested in a DNA topoisomerase II cleavage assay to determine if the compounds function as poisons. The results show that the acridines synthesized in this study function through a different mechanism to that of amsacrine, most likely by blocking topoisomerase binding to DNA (akin to that of aclarubicin). This not only suggests a unique mechanism of action in treating herpes virus infections, but also may be of great interest in the development of anticancer agents that target topoisomerase II activity.
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1. Introduction

The herpes virus family consists of more than 100 known viruses that affect practically all vertebrates including humans.[1] Some of the most notable herpes viruses affecting humans include HSV-1, HSV-2, VZV, HCMV, HHV-6, HHV-7, EBV, and HHV-8. Although most of these viruses are self-limiting, they remain significant human pathogens causing a diverse set of diseases ranging from cold sores to life-threatening illness.[2,3] Even the most benign infections pose serious danger in immunocompromised individuals, such as in AIDS or chemo-suppressed organ transplant patients.[1,2] Numerous studies over the past 50 years have focused on nucleoside-based anti-herpes agents with acyclovir being a excellent success story. However, modest advancement has been made since the beginning of the 1980s when acyclovir was introduced into clinical trials.[4] Now, with reports of acyclovir-resistant herpes strains emerging, the need for new alternative anti-herpes agents is even greater.[3–5]

Herpes viruses consist of a single piece of linear double-stranded DNA ranging from 120 to 230 kbp in size. The DNA is packaged within an icosahedral capsid approximately 100 nm in diameter. The capsid is further surrounded by an amorphous layer of at least 15 proteins and a glycoprotein-containing envelope. The herpes viruses have a common replication cycle with large DNA genomes that encode for more than 50 proteins which are involved in multiple stages in the virus life cycle.[3] Although herpes viruses encode for numerous replication enzymes, the viruses also require several from the host for normal function. For example, herpes viruses are known to utilize such enzymes as topoisomerase (topo) I and II, DNA ligase I, and DNA polymerase-α primase.[1] Of these host enzymes, topo I and topo II have received the most attention as possible targets for anti-herpes therapy.[6–14] However, the discovery of compounds that effectively inhibit viral replication as a result of topo inhibition has been limited.

Topoisomerase alters DNA topology through the decatenation and relaxation of supercoiled DNA.[15] By unwinding double-stranded DNA, this essential enzyme

Keywords: Heterocycle; Herpes virus; Antiviral; Topoisomerase; Acridine; Xanthone; Acridone; Acalrubicin; Amsacrine; Topoisomerase poison; Topoisomerase catalytic inhibitor; HSV-1; HSV-2; DNA binding; Intercalator.
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allows for normal cellular functions such as replication and transcription to occur.[15] Two types of topoisomerases exist, type I and type II. Type I functions by cutting one strand of the double-stranded DNA to unwind the duplex. Conversely, type II unwinds the DNA duplex by cutting both strands.[16] Within these two types of topoisomerases, the enzyme exists in various eukaryotic and prokaryotic forms.[17] Each variety may differ slightly in function by relaxing positive and/or negative supercoiling, introducing supercoiling, or decatenating positive or negative supercoiling.[17]

Two types of topo II inhibitors are known, which are the catalytic inhibitors and poisons.[8,9,15,18,19] Catalytic inhibitors interfere with any step of the topoisomerase catalytic cycle except for the stabilization of the covalent linkage between the DNA duplex and enzyme.[8,9,15] Steps inhibited may include DNA binding, ATP binding or hydrolysis, magnesium binding, or non-covalent linkages between DNA and enzyme.[15] In contrast, topo II poisons specifically stabilize the covalent topo II-5′phosphotyrosyl DNA intermediate, which is also called the cleavable complex.[15,18] Moreover, topo II poisons can be further subclassified into two groups: non-intercalating compounds, such as etoposide, and intercalators, such as amsacrine and doxorubicin.[18]

Intercalators act by forming ternary complexes with topo II and DNA, which then become trapped in the 'cleavable complex,' inhibiting the re-ligation process. Typically consisting of polyaromatic rings, intercalators associate with the nucleotide bases of the DNA helix and are stabilized through π stacking interactions.[20,21] However, the selectivity of intercalators to a particular DNA sequence is very low. Most often, selectivity is obtained from interactions of side-chain substitutions in the major and minor grooves.[20] These interactions between the drug and DNA disrupt the superhelical DNA structure, which may interfere with protein recognition.[21] Consequently, this may lead to cellular malfunction and disruption of cellular replication.

Aminoacridines have been used as antimicrobials for many years.[22,23] The general numbering scheme for the acridine core tricycle is indicated in Figure 1. Amsacrine (**2**) is a known 9-aminoacridine intercalator and topo II poison, but has not been associated with HSV inhibition. Although several studies have indicated that acridines have anti-herpes and anti-HIV activity, these studies do not point to a clear mechanism of action. However, the consensus is that the acridine analogs target DNA through intercalation and disrupt enzyme recognition and/or association.[24–29] A study by Vance and Bastow described a dihydroxyacridone (**3**) with anti-herpes activity in conjunction with topo II inhibition.[7] Additional studies involving synthetic acridones have demonstrated anti-herpes and anti-HIV activity although they have not indicated topo II as the target.[30–34] Lastly, the natural acridone alkaloid citrusinine-I (**4**) has demonstrated inhibition of both HSV-1 and HSV-2 infectivity.[35] To the best of our knowledge, there are no reports indicating that xanthones have antiviral activity.

In the current study, a series of acridones, xanthones, and acridines are investigated as potential anti-herpes agents. Compounds are screened in whole cell virus-induced cytopathogenic assays to determine EC_{50}s for both HSV-1 and HSV-2 inhibition. The cytotoxicity of these compounds to Vero cells is also assessed in the viral assays and in MTT cell viability assays. To investigate the mechanism of action for these compounds, topo II screening and DNA binding experiments are performed. Anti-herpes and topo II inhibition results are compared to determine the role that topo II has in the activity of these compounds. Finally, comparisons are made to known drugs that target topo II activity to gain insight into the structural basis of activity.

2. Synthesis

As shown in Scheme 1, the synthesis of acridones substituted in the 3-position began with a modified Ullmann–Goldberg coupling between bromoterephthalic acid (**5**) and aniline, producing the anthranilic intermediate **6**.[36,37] This reaction was catalyzed by copper, with CuI and pyridine as co-catalysts responsible for maintaining the catalytic cycle of Cu^0, reducing the reaction time, and increasing yields. In order to synthesize acridones substituted in the 2- and 4-positions, different starting reagents were required in the Ullmann–Goldberg coupling. Hence, the reaction between 2-chlorobenzoic acid (**7**) and the appropriate aminobenzoic acid **8** generated the desired substitutions. It should be noted that derivatives substituted in the 3-position cannot be made from 2-chlorobenzoic acid and 3-aminobenzoic acid because the reaction yields a mixture of 1- and 3-substituted products, which cannot be separated easily. Cyclization of the anthranilic intermediate **6** or **9** with $POCl_3$ generated the 9-chloroacridine intermediate **10** in addition to

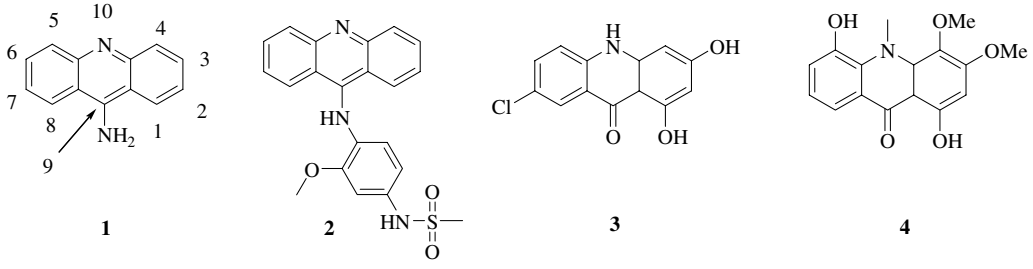
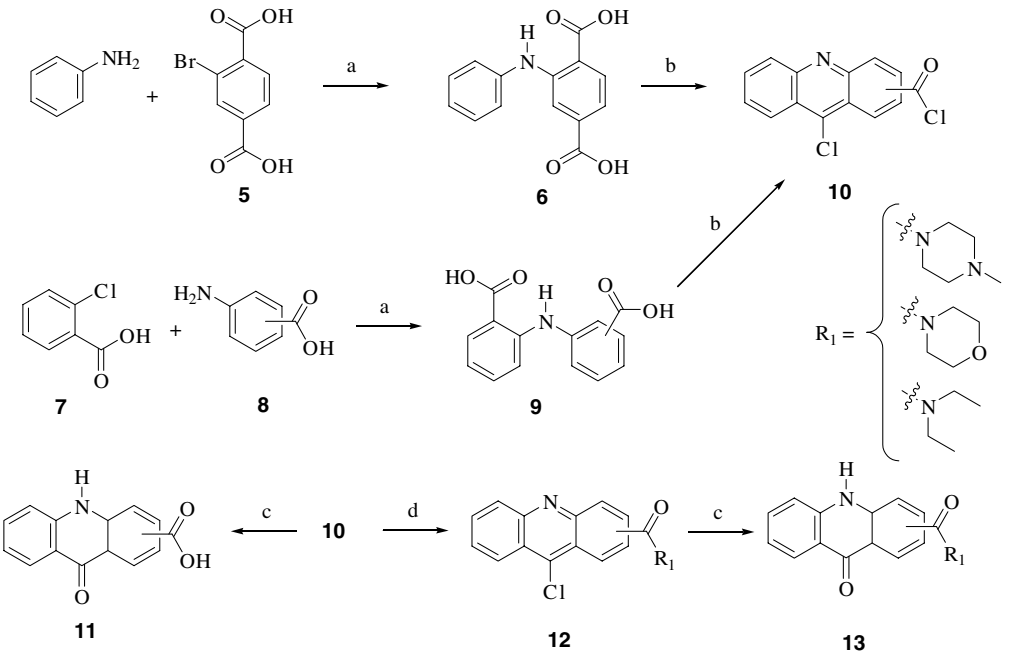
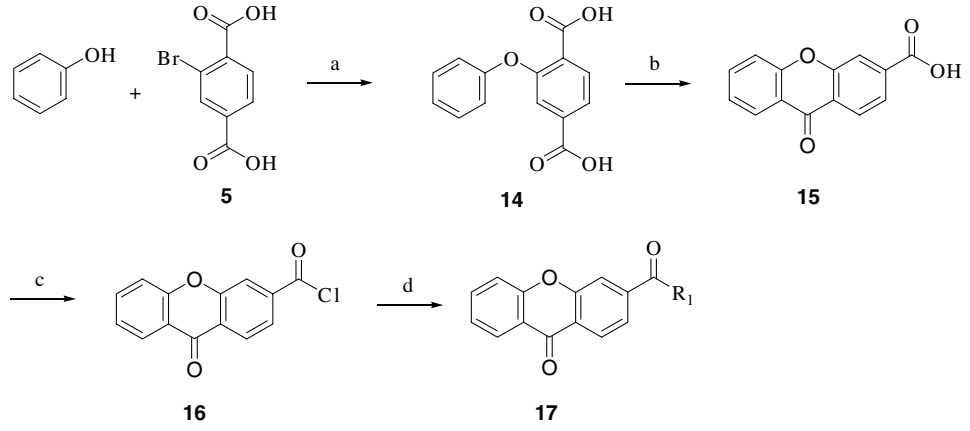


Figure 1. Structures of known acridines and acridones.



Scheme 1. Synthesis of acridone compounds. Reagents and conditions: (a) Cu, CuI, pyridine, K_2CO_3, H_2O, reflux; (b) $POCl_3$, reflux; (c) 1 M HCl, rt; (d) amine, CH_2Cl_2, 0 °C.



Scheme 2. Synthesis of xanthane compounds. Reagents and conditions: (a) phenol, Cu, CuI, pyridine, DBU, DMF, reflux; (b) H_2SO_4, 100 °C; (c) $SOCl_2$, reflux; (d) amine, CH_2Cl_2.

producing an acid chloride of the second carboxylic acid.[23] This intermediate was then hydrolyzed in 1 M HCl yielding the carboxyacridone **11**. Alternatively, intermediate **10** was reacted with an amine yielding the unstable 3-carboxamide-9-chloroacridine intermediate **12**. Subsequent hydrolysis with 1 M HCl produced the 3-carboxamideacridone **13**.

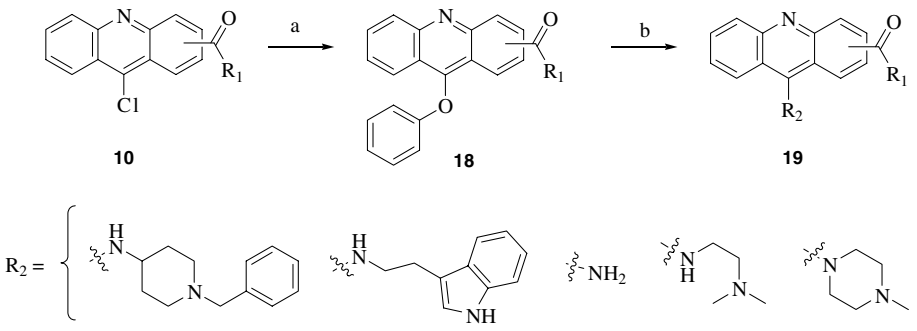
The synthesis of xanthones follows a similar synthetic route as the acridones, as shown in Scheme 2. The first step was also a modified Ullmann–Goldberg coupling between bromoterephthalic acid (**5**) and phenol. The use of a non-nucleophilic base, such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), was essential for coupling with phenol to form the *O*-phenylsalicylic acid **14**. Cyclization of **14** with concentrated H_2SO_4 at 100 °C yielded the 3-carboxyxanthone (**15**). Refluxing with $SOCl_2$ produced the acid chloride intermediate **16**. Subsequent reaction of the acid chloride with an amine afforded the 3-carboxamidexanthone **17**.

Acridine derivatives were synthesized from the 9-chloroacridine intermediate **10** shown in Scheme 1. The reaction of this intermediate with phenol under mild heating produced the stable 9-phenoxyacridine **18** as shown in Scheme 3. The final step entailed reacting the phenoxy intermediate **18** with the desired amine producing the substituted 9-aminoacridine **19**.[38]

3. Results and discussion

3.1. Anti-herpes activity

The HSV-1 and HSV-2 virus-induced cytopathogenic results for the acridone-, xanthone-, and acridine-based



Scheme 3. Synthesis of acridine compounds. Reagents and conditions: (a) phenol, 60 °C; (b) amine, 120 °C.

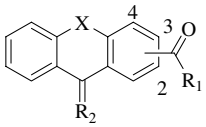
compounds are reported in Table 1. The EC_{50} values for compounds demonstrating activity greater than 50 μM were approximated from the initial screening, while compounds with better activity were subsequently tested in smaller increments around the approximated EC_{50} values. Also included in Table 1 are the Vero cell cytotoxicities for the compounds in this study and the resulting therapeutic indices (TI), where applicable. It was found that the acridone compounds bearing substituted carboxamides in the 2-, 3-, and 4-positions exhibited moderate activity selective for HSV-1, ranging from approximately 80 to 160 μM. These are interesting results since there have not been previous reports of acridones demonstrating reasonable selectivity between HSV-1 and HSV-2. It should be noted, however, that the marginal activities and poor TI values (ranging from 1.9 to 3.8) associated with these compounds could, in part, prevent the observation of activity against HSV-2. Nevertheless, it is apparent that the carboxamide functionalities play some role in the anti-HSV-1 activities since the carboxy analogs lack comparable activities. In contrast, the 9-aminoacridines bearing the same substituted carboxamides exhibited non-selective activity for both HSV-1 and HSV-2, ranging from 14.2 to 42.8 μM, as shown in Table 1. The results for the only two 9-aminoacridines with a morpholino-containing carboxamide (compounds **19b** and **19e**) did not show anti-herpes activity. Although the morpholino-containing carboxamides were only obtained in the 4-position of the acridine ring, it is believed that similar results would be seen in the 3- and 2-positions as well. In addition, no activity was found when the 9-position was substituted with a phenoxy group, suggesting that the 9-amino functionality may be important for activity.

A closer look at the results for the 9-aminoacridines shows that the diethylamine-containing carboxamides in the 2- and 3-positions exhibit the greatest degree of activity. These trends are similar for both the benzylpiperidinyl and tryptamino substitutions. To investigate the importance of the aromatic moieties at the 9-position of these compounds, three additional 9-aminoacridines were synthesized and screened, including a 9-amino (**19o**), a 9-(*N*,*N*-dimethylethylenediamino) (**19p**), and a 9-(*N*-methyl-piperazinyl) (**19q**). While none of these compounds showed activity in the herpes assays, **19p** and **19q** did show toxicities similar to the acridones. This observation, coupled with previous reports of acridine hydrolysis mechanisms, led to a computational and spectrophotometric investigation of the stability of the 9-aminoacridines **19l**, **19o**, **19p** and **19q**.[39] It was determined that under assay conditions (i.e., temperature and pH), both **19p** and **19q** were highly prone to hydrolysis to the acridone structure, exhibiting effective half-lives of 2.6 h and 3.3 h, respectively. In contrast, compounds **19l** and **19o** were deemed stable with half-lives significantly greater than 144 h. The lack of activity for **19o** suggests that the 9-N-substitution plays some role in activity, as does the nitrogen in the 10-position of the heterocyclic ring (as evident from the lack of activity of the xanthones).

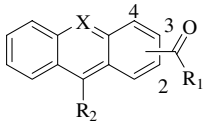
3.2. Topoisomerase activity

The intercalation of substituted 9-aminoacridines in nucleic acid structures, in particular those bearing substituted carboxamides, has been well documented using X-ray crystallography and DNA binding studies.[20,40–44] In addition, numerous studies have made the connection between 9-aminoacridine intercalators, such as amsacrine, and the inhibition of topoisomerase.[18,42,45] As discussed previously, the herpes viruses require host enzymes, such as topo II, for viral DNA replication. As a result, numerous studies have suggested topo II as a potential target of herpes inhibition. To determine if the 9-aminoacridines which exhibited anti-herpes activity in the current study were targeting topo II, they were first screened for inhibition of topo II relaxation activity. From the results shown in Figure 2A–C, it is apparent that a number of these compounds demonstrate observable inhibition of topo II relaxation. The apparent lack of inhibition for several 9-aminoacridines could be due, in part, to the concentration of compound tested or more likely to the probability that not all of the analogs share the same DNA binding site. Since acridine compounds of this type are not believed to intercalate at a specific site,[20,21] it is also possible that these compounds inhibit other host enzymes, such as topo I, which is known to recognize a different DNA sequence than topo II. The important observation at this point in time is that 9-aminoacridines showing anti-herpes activity also show inhibition of topo II-mediated relaxation of scDNA. In contrast, the acridone compounds show no activity in the topo II relaxation assay, as shown in Figure 2D (at concentrations up to 250 μM). While this is consistent with the low antiviral activity of these compounds, the results are somewhat surprising

Table 1. HSV-1, HSV-2, and toxicity results for the acridone, xanthone, and acridine series of compounds. HSV-1 and HSV-2 EC_{50}s and CC_{50} results are reported in μM[a]



Compounds **11a - 17c**



Compounds **18a - 19q**

Compound	X	Ring sub	R_1	R_2	HSV-1	HSV-2	CC_{50}[b]	TI[c]
11a	NH	4	Hydroxyl	Keto	>300	>300	>300	—
13a	NH	4	*N*-Methylpiperazinyl	Keto	80	>300	>300	3.8
13b	NH	4	Morpholino	Keto	80	>300	>300	3.8
13c	NH	4	Diethylamino	Keto	80	>300	>300	3.8
11b	NH	3	Hydroxyl	Keto	>300	>300	>300	—
13d	NH	3	*N*-Methylpiperazinyl	Keto	160	>300	>300	1.9
13e	NH	3	Morpholino	Keto	160	>300	>300	1.9
13f	NH	3	Diethylamino	Keto	80	>300	>300	3.8
11c	NH	2	Hydroxyl	Keto	>300	>300	>300	—
13g	NH	2	*N*-Methylpiperazinyl	Keto	80	>300	>300	3.8
13h	NH	2	Morpholino	Keto	80	>300	>300	3.8
13i	NH	2	Diethylamino	Keto	80	>300	>300	3.8
15	O	3	Hydroxyl	Keto	>300	>300	>300	—
17a	O	3	*N*-Methylpiperazine	Keto	>300	>300	>300	—
17b	O	3	Morpholino	Keto	>300	>300	>300	—
17c	O	3	Diethylamino	Keto	>300	>300	>300	—
18a	N	4	*N*-Methylpiperazinyl	Phenoxy	>92.8	>92.8	92.8	—
18b	N	4	Morpholino	Phenoxy	>102.6	>102.6	102.6	—
18c	N	4	Diethylamino	Phenoxy	>200[d]	>200[d]	>200[d]	—
19a	N	4	*N*-Methylpiperazinyl	4-Amino-*N*-benzylpiperdinyl	35.4	30.4	71.8	1.7
19b	N	4	Morpholino	4-Amino-*N*-benzylpiperdinyl	>91	>91	91.0	—
19c	N	4	Diethylamino	4-Amino-*N*-benzylpiperdinyl	42.8	37.5	62.6	1.5
19d	N	4	*N*-Methylpiperazinyl	Typtamino	30.0	29.8	52.5	2.0
19e	N	4	Morpholino	Typtamino	>64.7	>64.7	64.7	—
19f	N	4	Diethylamino	Typtamino	40.1	28.6	37.5	0.9
18d	N	3	*N*-Methylpiperazinyl	Phenoxy	>98.9	>98.9	98.9	—
18e	N	3	Diethylamino	Phenoxy	>200[d]	>200[d]	>200[d]	—
19g	N	3	*N*-Methylpiperazinyl	4-Amino-*N*-benzylpiperdinyl	17.2	18.3	39.1	2.3
19h	N	3	Diethylamino	4-Amino-*N*-benzylpiperdinyl	16.1	15.9	39.2	2.4
19i	N	3	*N*-Methylpiperazinyl	Tryptamino	37.7	48.5	38.5	1.0
19j	N	3	Diethylamino	Tryptamino	17.2	15.0	29.5	1.7
18f	N	2	*N*-Methylpiperazinyl	Phenoxy	>97.7	>97.7	97.7	—
18g	N	2	Diethylamino	Phenoxy	>200[d]	>200[d]	>200[d]	—
19k	N	2	*N*-Methylpiperazinyl	4-Amino-*N*-benzylpiperdinyl	24.2	22.2	49.9	2.1
19l	N	2	Diethylamino	4-Amino-*N*-benzylpiperdinyl	15.1	14.2	40.1	2.6
19m	N	2	*N*-Methylpiperazinyl	Tryptamino	26.5	25.0	38.1	1.4
19n	N	2	Diethylamino	Tryptamino	17.2	19.5	38.1	2.2
19o	N	2	Diethylamino	Amino	>300	>300	>300	—
19p	N	2	Diethylamino	*N*,*N*-Dimethylethylenediamino	>300	>300	>300	—
19q	N	2	Diethylamino	*N*-Methyl-piperazinyl	>300	>300	>300	—
Acyclovir	—	—	—	—	0.44	0.44	>300	>680

[a] EC_{50}s greater than 50 μM were approximated from the initial broad screen. EC_{50}s lower than 50 μM were subjected to a narrow screen around the approximate EC_{50} in order to obtain more accurate values.
[b] CC_{50}s were measured using a MTT cell viability assay.
[c] TI values were calculated by the equation (TI = CC_{50}/HSV-1 EC_{50}s).
[d] Compound precipitated out under assay conditions.

given the work of Bastow and co-workers. Using similar topo II relaxation assays, that group has reported topo II activity for two 1,3-dihydroxyacridone derivatives with low micromolar anti-herpes activity.[7] The compounds studied by Bastow and co-workers, however, are less substituted (and more planar) than those reported here and may in fact function through a different mechanism.

To further elucidate the exact site in the catalytic cycle that the 9-aminoacridines are inhibiting, compounds were screened in a cleavable complex assay. This assay evaluates inhibition of topo II through compound-induced stabilization of the DNA–enzyme cleavable complex. Compounds that function in this manner cause an increase in intensity of a linear DNA fragment, as described in Figure 3, and are commonly referred to

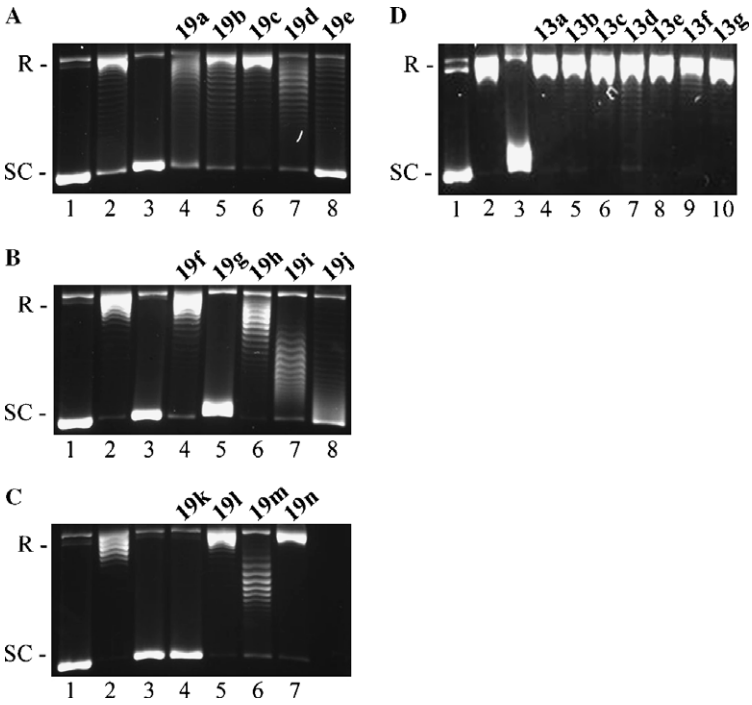


Figure 2. Topo II relaxation assay results for the acridine (A–C) and acridone (D) series of compounds. R, relaxed DNA; SC, supercoiled DNA. Lane 1 is the SC DNA marker, lane 2 is topo II relaxation control, and lane 3 is AMSA (200 μM) positive inhibition control.

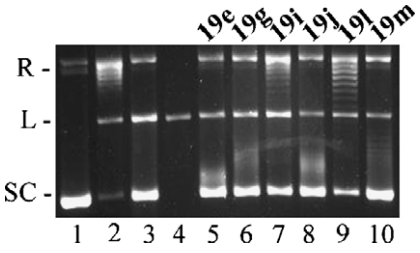


Figure 3. Results for a selection of six 9-aminoacridines. R, relaxed DNA; SC, supercoiled DNA; L, linear DNA. Lane 1 is the SC DNA marker, lane 2 is the topo II relaxation control, lane 3 is the amsacrine (200 μM) positive inhibition control, and lane 4 is the linear marker.

as poisons. The presence of a linear DNA fragment in the topo II relaxation control lane is an artifact due to the manner in which the reaction must be conducted in order to observe cleavable complex formation. By stopping the reaction with SDS (which denatures the enzyme) the catalytic cycle is completely stopped preventing further functional enzymatic activity, and thus, DNA is prevented from re-ligating. Only one compound, **19i**, appears to show an increase in the linear band intensity, but also shows significant relaxation of DNA. The remaining compounds in the series tested show no increase in the amount of linear DNA as compared to the amount in the enzyme control (lane 2). These results indicate that the acridines do not inhibit topo II activity through stabilization of the cleavable complex and therefore do not function as poisons like amsacrine (also shown in Fig. 3).

The most plausible explanation for the inhibition of topo II by the 9-aminoacridines in the current study is the blocking of topo II association with DNA in the first step of the catalytic cycle, by partially occupying either the major or minor groove. As shown in Figure 4, the compound aclarubicin (**21**), a larger analog of the topo II poison doxorubicin (**20**), inhibits topo II in this manner.[15] With the incorporation of two additional sugar moieties in aclarubicin, topo II is not able to bind to DNA and thus, the observation of the cleavable complex is not possible. It is therefore our current hypothesis that 9-aminoacridines containing the benzylpiperidinyl or tryptamino groups function by a similar mechanism. Structural comparisons of 9-aminoacridines from the current study with amsacrine are also shown in Figure 4.

3.3. Evaluation of compound–DNA binding

To evaluate the DNA binding affinity of the compounds reported in Table 1 (in the absence of topo II), ethidium bromide displacement experiments were carried out using calf thymus DNA. This is a useful technique for indicating if compounds bind DNA based on the enhanced fluorescence of DNA-bound verses unbound ethidium bromide.[46–48] Previous work has shown that there is a 50-fold increase in fluorescence when ethidium bromide is bound to DNA. This increase is measured by monitoring the emission at 595 nm while exciting at 546 nm using a spectrofluorimeter.[48] The concentration of compound required to reduce the enhancement in fluorescence by 50% is reported as C_{50}.[47] The results reported in Table 2 indicate that the acridines (**19a**–**19n**) bind DNA with C_{50}s ranging from 2 to 96 μM, suggesting these compounds have significant affinity for DNA. None of the acridone compounds, however, were found to displace ethidium bromide in this assay. The compounds were also assayed for fluorescence quenching to verify that the DNA binding results are due to

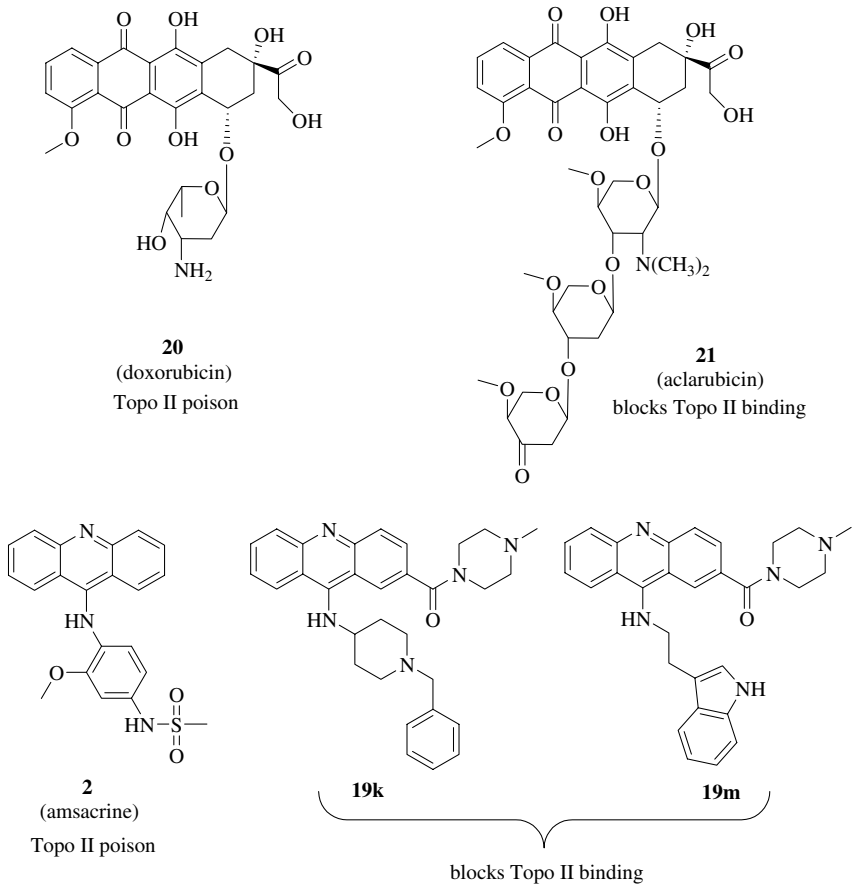


Figure 4. Inhibitors of topoisomerase II and mechanisms of inhibition.

Table 2. DNA binding values and percentage of fluorescence quenching for acridine and acridone-based compounds

Compound	C_{50}[a] (μM)	Q[b]
19a	6	6.8
19b	44	1.8
19c	72	0.0
19d	26	1.7
19e	96	0.3
19f	75	0.4
19g	2	7.5
19h	14	3.8
19i	11	6.4
19j	60	4.6
19k	2	8.3
19l	22	6.0
19m	10	7.1
19n	67	0.0
13a–13i	>300	—

[a] C_{50} values are defined as the concentration of compound required to reduce fluorescence of ethidium bromide bound to DNA by 50%.
[b] Percentage of fluorescence quenching of ethidium bromide bound to DNA by compound below saturation conditions.

the displacement of ethidium bromide and not from the reduction of fluorescence due to quenching effects, as shown in Table 2.[46] Control experiments were also conducted to insure that the acridine derivatives did not affect the fluorescence of free ethidium bromide and that acridine-bound DNA did not show fluorescence under the conditions of the assay.

4. Conclusion

This study has examined several substituted acridines and acridones to gain insight into the mechanism of action and structural features of the compounds that may lead to anti-herpes activity. Given the long history and wealth of information regarding acridines and their ability to intercalate into DNA, it is most probable that the acridine analogs function through the disruption of enzyme/DNA interactions. Our data support this general hypothesis and suggest a link may exist between the anti-herpes activity of substituted acridines and catalytic inhibition of topo II. It is important to point out, however, that not all of the acridine derivatives that show anti-herpes activity were found to be active in the topo II relaxation assay. This is most likely due to lack of specificity in DNA binding and intercalation. While the 9-aminoacridine compounds are believed to generally prefer intercalation into GC-rich sequences,[21,26,28] intercalation into a specific region of DNA is highly unlikely due to the vast size of the DNA genome and numerous GC-rich regions. Furthermore, the probability of all acridine analogs intercalating into a region recognized by a particular enzyme such as topo II may be small. Currently, we suspect that structural modifications to the acridine analogs alter either the electronic character of the acridine tricycle or the steric fit of the compound, causing the DNA binding preferences to shift. If this shift prevents the compounds from interca-

lating into a region recognized by topo II, then inhibition of the enzyme is simply not observed. This would suggest that other required replication enzymes such as topo I, which recognize a different sequence than topo II, are also targeted by these compounds. This general conclusion is further supported by the DNA binding results that indicate that the acridine compounds bind DNA in the absence of topo II enzyme.

One of the more surprising results of this study was the apparent lack of activity of the acridone compounds in the topo II relaxation assay. Previous work by Bastow and co-workers has suggested that two 1,3-dihydroxyacridone derivatives were active in similar assays and function through interactions with the topo enzyme, inhibiting catalytic activity. Since the acridones studied here were less active than those reported by Bastow and co-workers, the disparity may simply be the result of weak binding. It is also important to point out that our compounds contain larger steric substitutions, which may have a significant impact on function as well. Nevertheless, the lack of DNA binding character of these compounds is consistent with an alternative mechanism of action (as compared to the acridines). While the results are provocative, further work is required to understand the structure–function relationships of the acridones studied here and elsewhere before any definitive conclusions can be reached.

Finally, the finding that the acridines may function through non-specific binding, such as intercalation, presents a significant challenge to the development of useful drug leads. While the diethylamine-containing carboxamides in the 2- and 3-positions tend to show the greatest activity (e.g., **19h**, **19j**, **19l**, and **19n**), the activities are all in the low micromolar range. This is true for nearly all active compounds in the series, rendering SAR studies problematic at best. This problem is further echoed by the high toxicity and low therapeutic index of the compounds. Since these compounds are most likely targeting a host enzyme, such as topo II, it appears that cytotoxicity is an inherent problem linked to their activity. Therefore, the ability to produce compounds with greater anti-herpes activity via increased topo II inhibition is a formidable task. Nevertheless, acridines bearing substituted carboxamides and bulky 9-amino functionalities are able to inhibit herpes infections. Interestingly, when amsacrine was tested for anti-herpes activity in this study (results not shown), it was found to have substantial toxicity, preventing the observation of anti-herpes activity, even at nM concentrations. This seems to suggest that increased topo II inhibition will certainly lead to increased toxicities. Although amsacrine functions as a topo II poison, it is important to point out that aclarubicin (a potent antitumor agent) is an inhibitor of topo II relaxation.[15,49] The finding that the substituted acridines may share a similar mechanism to aclarubicin is extremely provocative and suggests that our antivirals may also be useful as anti-cancer agents. Moreover, comparisons of the acridines with aclarubicin may provide much needed insight into the design of more potent topo II inhibitors that may have both antiviral and anticancer properties.

5. Experimental

5.1. Biological general

Vero cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). HSV-1 and HSV-2 virus stocks were also obtained from ATCC (Manassas, VA). Human DNA topoisomerase II (p170 form) was purchased from TopoGen Inc. and came in 2 or 4 U/μL. Biological supplies were purchased from known suppliers including Sigma–Aldrich Chemical Co. (Milwaukee, WI), Fisher Scientific Co. (Hampton, NH), VWR Scientific Co. (West Chester, PA), etc. Cells and virus were maintained at 37 °C in a humidified 5% CO_2 atmosphere in a Fisher Scientific Model 5 Water Jacketed Carbon Dioxide Incubator. The supercoiled DNA utilized was obtained from rat opioid receptor cDNA, which was then subcloned into pcDNA3 (Invitrogen). In addition, amsacrine was obtained from Sigma–Aldrich.

5.2. Herpes assay

In vitro antiviral assays were carried out by virus-induced cytopathogenic effect (CPE) inhibition studies. Confluent monolayers of African green monkey cells (Vero cell line) grown in minimal essential media supplemented with 5% fetal calf serum in Falcon 96-well tissue culture plates were infected with an innoculum of HSV-1 (strain F) or HSV-2 (strain G). Initial screens were conducted at final concentrations of 100 μg/mL followed by 10-fold serial dilutions of the test compounds for a total of four test concentrations. Each test concentration was added to the 96-well plate and was tested in triplicate. Compounds showing either significant activity or substantial toxicity required the use of additional dilutions and were tested in triplicate twice. Some wells were used as virus controls and left free of test compound. Other wells remained free of virus to determine preliminary drug cytotoxicity to Vero cells. Acyclovir was used as a positive control. The 96-well plates were incubated for 3 days at 37 °C in a humidified atmosphere containing 5% CO_2 until a maximum CPE (100% infected) was observed in the virus control cultures. The cell monolayers were examined microscopically for virus-induced CPE and for preliminary drug cytotoxicity. The EC_{50} values of the inhibitors are defined as the concentration of drug that decreased the number of plaque-covered area by 50% of that present in the compound-free virus control wells. Examples of assay controls illustrating the different cell morphologies that were microscopically observed are shown in Figure 5.

5.3. MTT cell viability (cytotoxicity) assay

Compound in DMSO was added to minimal essential media supplemented with 5% fetal calf serum followed by serial dilution with media. The serially diluted compound was then added to 96-well plates (200 μL/well)

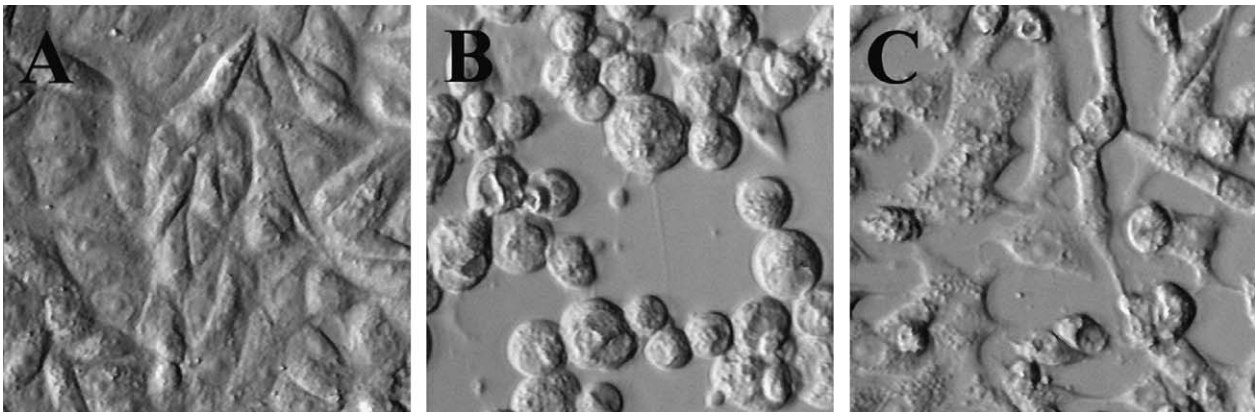


Figure 5. Sample images of different morphological states of Vero cells grown for three days under various conditions. (A) Cell control, no virus and no compound. (B) Virus control, cells infected with HSV-2 only. (C) Toxicity control, cells only treated with compound. This example is from compound **19l** at a toxic concentration of 150 μM. In (A), cells appear healthy (alive) and are confluent. In (B), cells now appear round and bloated, ready to rupture. In (C), cells are not confluent and a number of dead cells are shown.

containing a confluent monolayer of Vero cells and was incubated for 71 h (1 h short of 3 days) at 37 °C in a humidified atmosphere containing 5% CO_2. The media containing compound were then removed from the 96-well plates followed by a single rinse with phosphate-buffered saline. RPMI media without phenol red (100 μL) containing 0.83 mg/mL MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) were then added to each well and incubated for an additional hour, at which time purple precipitate is visual. MTT-containing media was then removed from the 96-well plates followed by another rinse with phosphate-buffered saline. Next, DMSO (100 μL) was added and incubated for 1 h in order to dissolve the resulting intracellular purple formazan. The plates were then read at 570 nm on a BioTek SynergyHT 96-well plate reader. The results are reported as CC_{50}s which were determined by interpolation of data points conducted in triplicate.

5.4. Compound–DNA binding

C_{50} values for compound–DNA binding were determined using an adapted procedure. Measurement of fluorescence reduction of ethidium bromide bound DNA was conducted with a BioTek SynergyHT 96-well plate reader equipped with a 540 ± 20 nm filter for affecting excitation and a 590 ± 20 nm filter for monitoring emission. Stock solutions of compound were prepared in DMSO and serially diluted into DMSO to insure that the concentration of DMSO was held constant. Serial concentrations of compound were diluted into assay buffer containing ethidium bromide (1.26 μM) and calf thymus DNA (1 μM, based on average mass of nucleotide monophosphate; Sigma Chemical Co., St. Louis, MO), added to a black walled and bottomed 96-well plate, and allowed to equilibrate for 1 h at rt. The assay buffer was composed of 2 mM Hepes, 10 μM EDTA, and 9.4 mM NaCl. The pH of the assay buffer was adjusted to 7.0 with NaOH. All binding assays were conducted in duplicate.

5.5. Fluorescence quenching

The percentage of fluorescence quenching (Q-values) can be gauged under non-saturation conditions using a similar protocol as described for compound–DNA binding. In this assay, the concentration of calf thymus DNA is increased to 40 μM (based on average mass of nucleotide monophosphate) and the concentration of both ethidium bromide and test compound is 2 μM such that there was minimal displacement of ethidium bromide and any reduction in fluorescence can be attributed to quenching effect.

5.6. DNA topoisomerase II catalytic assay

In order to determine if topo II activity was inhibited, a relaxation assay of supercoiled pcDNA3 (scDNA) containing the KOR gene was conducted using a modified TopoGEN topoisomerase drug screening kit protocol. Final assay volumes of 20 μL were composed of 49.98 mM Tris (pH 8), 150 mM K-Glu, 10 mM $MgCl_2$, 10 mM DTT, 0.05 mg/mL BSA, 1 mM ATP, 5 μg/mL tRNA, 15 mM scDNA, and 250 μM of compound. Each relaxation assay consisted of the following control reactions: scDNA marker, topo II enzyme activity control, and positive inhibition control using amsacrine. All reactions were conducted at 1% DMSO final concentration and were prepared on ice. Reactions were initiated by adding 2 units of human DNA topo II and were immediately placed in an incubator for 30 min at 37 °C. Termination ensued with the addition of 1 μL of 0.5 M EDTA and was further incubated at 37 °C for 15 min. EDTA chelates magnesium, stopping the catalytic cycle in progress, preventing additional cycles and consequently, preventing further unwinding of the DNA. The absence of free magnesium initiates re-ligation and disassociation from the DNA, which results in striated DNA bands (topoisomers). From each reaction mixture, 15 μL was loaded onto a 1.2% agarose gel and then electrophoresed in TAE buffer for 15 h at 22 V/cm. Gels were then stained in ethidium bromide (0.5 μg/mL) for 1/2 h and destained for 1 h before photography using LabWorks software.

5.7. DNA topoisomerase II cleavage assay

To determine if the compounds act as topo II poisons, a cleavable complex assay was performed using a modified TopoGEN protocol. A positive result is indicated by the presence of an observable increase in linear band intensity as compared to enzyme control. Final assay volumes consisted of 20 μL containing 49.98 mM Tris (pH 8), 50 mM K-Glu, 10 mM $MgCl_2$, 10 mM DTT, 0.05 mg/mL BSA, 1 mM ATP, 5 μg/mL tRNA, 15 mM pcDNA3, and 250 μM of compound. Once again, all reactions were conducted at a final concentration of 1% DMSO and were prepared on ice. To initiate reactions, 8 units of human DNA topo II was added. Upon enzyme addition, reaction mixtures were incubated at 37 °C for 10 min. The reactions were terminated by the addition of 2.5 μL of 10% SDS and then further incubated at 37 °C for 10 min. SDS is required to observe a linear DNA fragment. As a result, a linear DNA fragment is present in the topo control. SDS denatures topo II, preventing further functional enzymatic activity, and thus DNA is prevented from re-ligating. Next, 2 μL of 0.5 M EDTA and 0.5 μL of 10 mg/mL proteinase K (digests protein) were then simultaneously added to the reaction mixture and were incubated at 37 °C for an additional 15 min. Reaction mixtures were then electrophoresed, stained, destained, and photographed in the same fashion as the topo II catalytic assay mixtures.

5.8. Chemistry general

All reagents were purchased from commercial suppliers such as Sigma–Aldrich Chemical Co., Lancaster Chemical Co, Acros Chemical Co., etc. ACS Reagent grade or better solvents were used without further purification unless otherwise noted. Water was purified by Millipore filtration system. Column chromatography was conducted using silica gel 60 (40–63 microns) and thin-layer chromatography was conducted using EMD Chemical silica gel 60 F_{254} on aluminum sheets. ^{1}H Nuclear magnetic resonance spectra were collected on a Varian Mercury 300 MHz instrument and a Varian Mercury 600 MHz instrument using $CDCl_3$ and DMSO-d_6 as solvents. High-resolution mass spectra (HRMS) were collected from a TOF-ESI Agilant LC–MS and analyzed using the Analyst QS software.

5.8.1. General procedure for phenylamino-terephthalic acid (6, 9a, and 9b)

5.8.1.1. 2-Phenylamino-terephthalic acid (6). Potassium carbonate (30.6 mmol) was first dissolved in 100 mL of distilled water. Next, 2-bromoterepthalic acid (**5**) (20.4 mmol) was added followed by aniline(40.8 mmol), pyridine (0.3 mL), copper(0) (0.1 g), and copper(I) iodide (0.1 g). The reaction mixture was stirred under reflux for 4 h. Once the reaction mixture was cooled, just enough 5% NaOH(aq) was added to completely dissolve the product. The mixture was then passed through a fritted filter containing a thin layer of silica gel between two layers of compressed celite and was rinsed with saturated $NaHCO_3$ to remove copper catalyst. The resulting filtrate was acidified using 3 M HCl(aq) until the pH 1. The resultant yellow precipitate (2-phenylamino-terephthalic acid) was then filtered and recrystallized from absolute ethanol/water. Yield: 64.7%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.09 (t, 1H), 7.27 (d, 2H), 7.41 (m, 1H), 7.82 (d, 1H), 7.95 (d, 1H), 8.13 (s, 1H), 9.60 (s, 1H).

5.8.1.2. *N*-(2-Carboxyphenyl)anthranilic acid (9a). Yield ~68%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.93 (m, 2H), 7.44 (m, 4H), 7.88 (m, 2H).

5.8.1.3. *N*-(4-Carboxyphenyl)anthranilic acid (9b). Yield ~43.1%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.92 (m, 1H), 7.26 (d, 2H), 7.48 (m, 2H), 7.85 (d, 2H), 7.93 (d, 1H), 9.79 (s, 1H).

5.8.2. General procedure for intermediate 10. 2-Phenylamino-terephthalic acid (**6**) (7.77 mmol) was dissolved in $POCl_3$ (~50 mL) and refluxed for 1 h. Excess $POCl_3$ was then recovered via vacuum distillation, being careful not to heat the reaction flask over 100 °C. The unstable product was used without further purification.

5.8.3. General procedure for intermediates 11a–c

5.8.3.1. 9-Oxo-9,10-dihydroacridine-4-carboxylic acid (11a). To a flask containing the 9-chloro intermediate **10** was added 30 mL of 1 M HCl. The reaction was mildly heated with a water bath while stirring for 2 h. The reaction was cooled and the product was extracted into CH_2Cl_2, dried over $MgSO_4$ and concentrated. The product was purified by reprecipitation from ethanol/water. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.33 (m, 2H), 7.76 (m, 2H), 8.22 (d, 1H), 8.43 (d, 1H), 8.51 (d, 1H), 11.94 (s, 1H); HRMS ($C_{14}H_{10}NO_3$) $[M+H]^+$: found *m*/*z* 240.0654, calcd 240.0661.

5.8.3.2. 9-Oxo-9,10-dihydroacridine-3-carboxylic acid (11b). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.27 (m, 1H), 7.58 (d, 1H), 7.72 (m, 1H), 7.79 (d, 1H), 7.94 (d, 1H), 8.13 (s, 1H) 8.28 (d, 1H), 12.09 (s, 1H); HRMS ($C_{14}H_{10}NO_3$) $[M+H]^+$: found *m*/*z* 240.0663, calcd 240.0661.

5.8.3.3. 9-Oxo-9,10-dihydroacridine-2-carboxylic acid (11c). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.29 (t, 1H), 7.65 (m, 2H), 7.76 (t, 1H), 8.18 (m, 2H), 8.81 (s, 1H), 12.51 (s, 1H); HRMS ($C_{14}H_{10}NO_3$) $[M+H]^+$: found *m*/*z* 240.0660, calcd 240.0661.

5.8.4. General procedure for intermediates 12. To intermediate **10** was added excess amounts of desired amine (example, *N*-methylpiperazine) (4 mL) dropwise while stirring. The reaction mixture was allowed to warm to room temperature then slightly heated while stirring for approximately 1 h. Then the reaction mixture was condensed and left under vacuum to remove residual amounts of amine. The unstable product was used without further purification.

5.8.5. General procedure for carboxyamide substituted acridones (13a–i)

5.8.5.1. 4-(4-Methylpiperazine-1-carbonyl)acridin-9(10*H*)-one (13a). To a flask containing 9-chloro inter-

mediate **10** was added 30 mL of 1 M HCl. The reaction mixture was mildly heated by water bath while stirring for 2 h. The reaction mixture was neutralized with ammonium hydroxide and extracted into CH_2Cl_2, dried over $MgSO_4$, and concentrated. The product was purified by column chromatography. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.19 (s, 3H), 2.28 (br s, 2H), 2.36 (br s, 2H), 3.33 (br s, 2H), 3.64 (br s, 2H), 7.27 (m, 2H), 7.60 (d, 1H), 7.71 (t, 1H), 7.89 (d, 1H), 8.21 (d, 1H), 8.30 (d, 1H), 10.70 (s, 1H); HRMS ($C_{19}H_{20}N_3O_2$) $[M+H]^+$: found *m*/*z* 322.1559, calcd 322.1556.

5.8.5.2. 4-(Morpholine-4-carbonyl)acridin-9(10*H*)-one (13b). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.22 (br s, 2H), 3.50 (br s, 2H), 3.75 (br s, 4H), 7.27 (m, 2H), 7.60 (d, 1H), 7.71 (t, 1H), 7.89 (d, 1H), 8.21 (d, 1H), 8.30 (d, 1H); 10.70 (s, 1H); HRMS ($C_{19}H_{17}N_3O_3$) $[M+H]^+$: found *m*/*z* 309.1229, calcd 309.1239.

5.8.5.3. *N*,*N*-Diethyl-4-acridin-9(10*H*)-onecarboxamide (13c). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.00 (br s, 3H), 1.23 (br s, 3H), 3.16 (br s, 2H), 3.61 (br s, 2H), 7.27 (m, 2H), 7.60 (d, 1H), 7.71 (t, 1H), 7.89 (d, 1H), 8.21 (d, 1H), 8.30 (d, 1H), 10.70 (s, 1H); HRMS ($C_{18}H_{19}N_2O_2$) $[M+H]^+$: found *m*/*z* 295.1456, calcd 295.1447.

5.8.5.4. 3-(4-Methylpiperazine-1-carbonyl)acridin-9(10*H*)-one (13d). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.19 (s, 3H), 2.28 (br s, 2H), 2.36 (br s, 2H), 3.33 (br s, 2H), 3.64 (br s, 2H), 7.19 (d, 1H), 7.26 (t, 1H), 7.47 (s, 1H), 7.53 (d, 1H), 7.74 (t, 1H), 8.22 (d, 1H), 8.25 (d, 1H), 11.81 (s, 1H); HRMS ($C_{19}H_{20}N_3O_2$) $[M+H]^+$: found *m*/*z* 322.1545, calcd 322.1556.

5.8.5.5. 3-(Morpholine-4-carbonyl)acridin-9(10*H*)-one (13e). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.22 (br s, 2H), 3.50 (br s, 2H), 3.75 (br s, 4H), 7.19 (d, 1H), 7.27 (t, 1H), 7.50 (s, 1H), 7.53 (d, 1H), 7.74 (t, 1H), 8.22 (d, 1H), 8.25 (d, 1H), 11.82 (s, 1H); HRMS ($C_{19}H_{17}N_3O_3$) $[M+H]^+$: found *m*/*z* 309.1232, calcd 309.1239.

5.8.5.6. *N*,*N*-Diethyl-3-acridin-9(10*H*)-onecarboxamide (13f). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.08 (br s, 3H), 1.16 (br s, 3H), 3.19 (br s, 2H), 3.46 (br s, 2H), 7.16 (d, 1H), 7.26 (t, 1H), 7.43 (s, 1H), 7.52 (d, 1H), 7.74 (t, 1H), 8.22 (d, 1H); 8.25 (d, 1H); 11.78 (s, 1H); HRMS ($C_{18}H_{19}N_2O_2$) $[M+H]^+$: found *m*/*z* 295.1440, calcd 295.1447.

5.8.5.7. 2-(4-Methylpiperazine-1-carbonyl)acridin-9(10*H*)-one (13g). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.19 (s, 3H), 2.33 (br s, 4H), 3.52 (br s, 4H), 7.28 (t, 1H), 7.55 (m, 2H), 7.75 (m, 2H), 8.20 (s, 1H), 8.22 (d, 1H), 11.91 (s, 1H); HRMS ($C_{19}H_{20}N_3O_2$) $[M+H]^+$: found *m*/*z* 322.1556, calcd 322.1556.

5.8.5.8. 2-(Morpholine-4-carbonyl)acridin-9(10*H*)-one (13h). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.53 (br s, 4H), 3.60 (br s, 4H), 7.28 (t, 1H), 7.56 (m, 2H), 7.75 (m, 2H), 8.20 (s, 1H), 8.23 (d, 1H), 11.92 (s, 1H); HRMS ($C_{19}H_{17}N_3O_3$) $[M+H]^+$: found *m*/*z* 309.1234, calcd 309.1239.

5.8.5.9. *N*,*N*-Diethyl-2-acridin-9(10*H*)-onecarboxamide (13i). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.13 (br s, 6H), 3.35 (br s, 4H), 7.24 (t, 1H), 7.55 (m, 2H), 7.73 (m, 2H), 8.16 (d, 1H), 8.21 (s, 1H), 11.90 (s, 1H); HRMS ($C_{18}H_{19}N_2O_2$) $[M+H]^+$: found *m*/*z* 295.1444, calcd 295.1447.

5.8.6. 2-Phenoxy-terephthalic acid (14). Bromoterephthalic acid (**5**) (12.4 mmol) was added to 100 mL dimethylformamide (DMF), followed by phenol (25.2 mmol), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (36.9 mmol), pyridine (0.2 mL), copper(0) (0.1 g), and copper(I) iodide (0.1 g). The reaction mixture was heated to reflux and monitored via TLC. After 2 h, TLC analysis showed that all the bromoterephthalic acid was consumed. The reaction mixture was cooled and diluted with 1 M HCl (~500 mL) until no more precipitate had formed. The resulting greenish solid was filtered and washed with water (~100 mL) and dried under vacuum. Yield: 93.5%; ^{1}H NMR (300 MHz, acetone-d_6) δ 7.05 (d, J = 7.77, 2H), 7.17 (t, 1H), 7.42 (t, 2H), 7.57 (d, J = 1.2 Hz, 1H), 7.88 (dd, J = 1.4 and 8.0 Hz, 1H), 8.03 (d, J = 8.0, 1H).

5.8.7. 9-Oxo-9*H*-xanthene-3-carboxylic acid (15). 2-Phenoxy-terephthalic acid (**14**) (10.6 mmol) was added to 100 mL H_2SO_4 and heated to 100 °C while stirring for 3 h. The starting material dissolved upon heating. The reaction mixture was cooled and poured over ice (300 mL) producing a gray solid which was then filtered and washed with water. Yield: 54.9%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.51 (t, 1H), 7.7 (d, 1H), 7.91 (t, 1H), 7.95 (d, 1H), 8.09 (s, 1H), 8.20 (d, 1H), 8.28 (d, 1H); HRMS ($C_{14}H_9O_4$) $[M+H]^+$: found *m*/*z* 241.0498, calcd 241.0500.

5.8.8. General procedure for 3-carboxamide substituted xanthone (17a–c)

5.8.8.1. 3-(4-Methyl-piperazine-1-carbonyl)-xanthen-9-one (17a). Thionyl chloride (~25 mL) was added to a flask containing 9-oxo-9*H*-xanthene-3-carboxylic acid (**15**) (1.08 mmol) and was set to reflux for 1 h. Excess thionyl chloride was distilled off under reduced pressure. The resulting residue was dissolved in CH_2Cl_2 (~15 mL) and cooled to 0 °C on an ice bath. Next, *N*-methylpiperazine (~2 mL) was added dropwise while stirring. The reaction mixture was allowed to reach room temperature under continued stirring for about 1 h. An additional 30 mL CH_2Cl_2 was added and the reaction mixture was extracted with 1 M HCl. The aqueous layer was made basic with 5% NaOH and extracted into EtOAc, dried with $MgSO_4$, and concentrated. Yield: 71.5%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.33 (s, 3H), 2.37 (br s, 2H), 2.52 (br s, 2H), 3.43 (br s, 2H), 3.83 (br s, 2H), 7.39 (m, 2H), 7.51 (m, 2H), 7.74 (t, 1H), 8.32 (d, 1H), 8.37 (d, 1H); HRMS ($C_{19}H_{19}N_2O_3$) $[M+H]^+$: found *m*/*z* 323.1426, calcd 323.1395.

5.8.8.2. 3-(Morpholine-4-carbonyl)-9*H*-xanthen-9-one (17b). Yield: 89.6%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.46 (br s, 2H), 3.67 (br s, 2H), 3.84 (br s, 4H), 7.41 (m, 2H), 7.54 (m, 2H), 7.77 (t, 1H), 8.35 (d, 1H), 8.40 (d, 1H);

HRMS ($C_{18}H_{16}NO_4$) $[M+H]^+$: found *m/z* 310.1076, calcd 310.1079.

5.8.8.3. *N,N*-Diethyl-9*H*-xanthen-9-one-3-carboxamide (17c). Yield: 72.4%; ^1H NMR (300 MHz, $CDCl_3$) δ 1.16 (s, 3H), 1.30 (br s, 3H), 3.28 (br s, 2H), 3.60 (br s, 2H), 7.40 (m, 2H), 7.52 (m, 2H), 7.76 (t, 1H), 8.33 (d, 1H), 8.38 (d, 1H); HRMS ($C_{18}H_{18}NO_3$) $[M+H]^+$: found *m/z* 296.1284, calcd 296.1287.

5.8.9. General procedure for intermediates 18

5.8.9.1. 4-(4-Methylpiperazine-1-carbonyl)-9-phenoxy-(10*H*)-acridine (18a). Intermediate **12** was placed under vacuum for 2 days to remove excess amine. Next phenol (~15 g) was added to the reaction flask and heated to 80 °C for 30 min with manual stirring using a thermometer. The reaction mixture was then cooled and diluted with 200 mL CH_2Cl_2. The mixture was then washed with 30% w/w NaOH in water (2× 150 mL) to remove phenol, washed with brine (150 mL), dried over $MgSO_4$, and concentrated. TLC analysis showed multiple spots, but the product is the major spot as seen by elution with 15:1 CH_2Cl_2/MeOH. Product was purified by silica chromatography eluting with 15:1. Fractions containing product were combined and concentrated. The resulting solid was dissolved in minimal amounts of EtOAc and precipitated out with petroleum ether or hexanes yielding the 9-phenoxy-acridine-3-carboxamide.Yield: 67.6%; ^1H NMR (300 MHz, $CDCl_3$) δ 2.22 (br m, 1H), 2.35 (s, 3H), 2.44 (br m, 1H), 2.60 (br m, 1H), 2.74 (br m, 1H), 3.24 (br m, 2H) 3.96 (br m, 1H), 4.16 (br m, 1H), 6.84 (d, 2H), 7.05 (t, 1H), 7.3 (m, 2H), 7.46 (t, 2H), 7.76 (m, 2H), 8.10 (t, 2H), 8.24 (d, 1H); HRMS ($C_{25}H_{24}N_3O_2$) $[M+H]^+$: found *m/z* 398.1836, calcd 398.1863.

5.8.9.2. 4-(Morpholine-4-carbonyl)-9-phenoxy-(10*H*)-acridine (18b). Yield: 45.1%; ^1H NMR (300 MHz, $CDCl_3$) δ 3.22 (m, 1H), 3.28 (m, 1H), 3.51 (m, 1H), 3.77 (m, 1H), 3.85 (m, 1H), 3.90 (m, 1H), 4.04 (m, 1H), 4.26 (m, 1H), 6.84 (d, 2H), 7.06 (t, 1H), 7.28 (t, 2H), 7.47 (m, 2H), 7.77 (m, 2H), 8.09 (d, 1H), 8.13 (d, 1H), 8.25 (d, 1H); HRMS ($C_{24}H_{21}N_2O_3$) $[M+H]^+$: found *m/z* 385.1529, calcd 385.1547.

5.8.9.3. *N,N*-Diethyl-9-phenoxy-(10*H*)-acridine-4-carboxamide (18c). Yield: 41.5%; ^1H NMR (300 MHz, $CDCl_3$) δ 0.96 (br s, 3H), 1.24 (br s, 3H), 3.21 (br s, 2H), 3.53 (br s, 2H), 6.84 (d, 2H), 7.05 (t, 1H), 7.3 (m, 2H), 7.46 (t, 2H), 7.76 (m, 2H), 8.10 (t, 2H), 8.24 (d, 1H).

5.8.9.4. 3-(4-Methylpiperazine-1-carbonyl)-9-phenoxy-(10*H*)-acridine (18d). Yield: 47.5%; ^1H NMR (300 MHz, $CDCl_3$) δ 2.34 (s, 3H), 2.41 (br s, 2H), 2.55 (br s, 2H), 3.58 (br s, 2H), 3.89 (br s, 2H), 6.87 (d, 2H), 7.07 (t, 1H), 7.28 (t, 2H), 7.50 (m, 2H), 7.81 (t, 1H), 8.11 (d, 1H), 8.16 (d, 1H), 8.25 (s, 1H), 8.27 (d, 1H); HRMS ($C_{25}H_{24}N_3O_2$) $[M+H]^+$: found *m/z* 398.1840, calcd 398.1863.

5.8.9.5. *N,N*-Diethyl-9-phenoxy-(10*H*)-acridine-3-carboxamide (18e). Yield: 55.5%; ^1H NMR (300 MHz, $CDCl_3$) δ 0.97 (br s, 3H), 1.26 (br s, 3H), 3.23 (br s, 2H), 3.54 (br s, 2H), 6.87 (d, 2H), 7.07 (t, 1H), 7.27 (t, 2H), 7.50 (m, 2H), 7.82 (t, 1H), 8.11 (d, 1H), 8.17 (d, 1H), 8.25 (s, 1H), 8.28 (d, 1H).

5.8.9.6. 2-(4-Methylpiperazine-1-carbonyl)-9-phenoxy-(10*H*)-acridine (18f). Yield: 41.3%; ^1H NMR (300 MHz, $CDCl_3$) δ 2.18 (br s, 2H), 2.30 (s, 3H), 2.46 (br s, 2H), 3.36 (br s, 2H), 3.79 (br s, 2H), 6.85 (d, 2H) 7.06 (t, 1H), 7.28 (m, 2H), 7.50 (m, 1H), 7.83 (m, 2H), 8.12 (m, 2H), 8.29 (t, 2H); HRMS ($C_{25}H_{24}N_3O_2$) $[M+H]^+$: found *m/z* 398.1837, calcd 398.1863.

5.8.9.7. *N,N*-Diethyl-9-phenoxy-(10*H*)-acridine-2-carboxamide (18g). Yield: 42.7%; ^1H NMR (300 MHz, $CDCl_3$) δ 0.96 (br s, 3H), 1.24 (br s, 3H), 3.21 (br s, 2H), 3.53 (br s, 2H), 6.85 (d, 2H) 7.06 (t, 1H), 7.28 (m, 2H), 7.50 (m, 1H), 7.83 (m, 2H), 8.12 (m, 2H), 8.29 (t, 2H).

5.8.10. General procedure for compounds 19a–q

5.8.10.1. 4-(4-Methylpiperazine-1-carbonyl)-9-(1-benzylpiperidin-4-ylamino)-(10*H*)-acridine (19a). 4-(4-Methylpiperazine-1-carbonyl)-9-phenoxy-(10*H*)-acridine (**14a**) (10 mmol) was dissolved in 2 mL of 4-amino-*N*-benzylpiperidine and heated to 120 °C (reflux) for 15 min. The reaction mixture was cooled and run on a silica column to purify the product and remove excess amine. A solvent ratio of 15:1 CH_2Cl_2/MeOH was used for the first 200 mL followed by 15:1:0.1 CH_2Cl_2/MeOH/NEt_3 until the product eluted from the column. The fractions with product were collected and concentrated. The yellow product was dissolved in EtOAc and precipitated out with petroleum ether or hexanes. Yield: 74.1%; ^1H NMR (600 MHz, $CDCl_3$) δ 1.74 (br s, 2H), 2.06 (br m, 4H), 2.18 (br s, 1H), 2.32 (s, 3H), 2.42 (br s, 1H), 2.58 (br s, 1H), 2.71 (br s, 1H), 2.88 (br s, 2H), 3.17 (br s, 1H), 3.26 (br s, 1H), 3.51 (s, 2H), 3.82 (br s, 1H), 3.89 (br s, 1H), 4.17 (br s, 1H), 4.65 (br s, 1H), 7.26 (m, 1H), 7.32 (m, 4H), 7.42 (m, 2H), 7.67 (m, 2H), 8.05 (d, 1H), 8.10 (m, 2H); HRMS ($C_{31}H_{36}N_5O$) $[M+H]^+$: found *m/z* 494.2876, calcd 494.2914.

5.8.10.2. 4-(Morpholine-4-carbonyl)-9-(1-benzylpiperidin-4-ylamino)-(10*H*)-acridine (19b). Yield: 60.7%; ^1H NMR (600 MHz, $CDCl_3$) δ 1.74 (br m, 2H), 2.07 (br m, 4H), 2.88 (br s, 2H), 3.17 (br s, 1H), 3.23 (br s, 1H), 3.46 (br s, 1H), 3.51 (br s, 2H), 3.82 (br m, 4H), 4.01 (br s, 1H), 4.26 (br s, 1H), 4.68 (br s, 1H), 7.26 (m, 1H), 7.32 (m, 4H), 7.42 (m, 2H), 7.67 (m, 2H), 8.05 (d, 1H), 8.10 (m, 2H); HRMS ($C_{30}H_{33}N_4O_2$) $[M+H]^+$: found *m/z* 481.2576, calcd 481.2598.

5.8.10.3. *N,N*-Diethyl-9-(1-benzylpiperidin-4-ylamino)-(10*H*)-acridine-4-carboxamide (19c). Yield: 17.7%; ^1H NMR (600 MHz, $CDCl_3$) δ 1.17 (br s, 3H), 1.30 (br s, 3H), 1.75 (br m, 2H), 2.07 (br m, 4H), 2.88 (br m, 2H), 3.41 (br s, 2H), 3.52 (s, 2H), 3.63 (br s, 2H), 3.87 (br s, 1H), 4.72 (br s, 1H), 7.26 (m, 1H), 7.34 (m, 4H), 7.44 (m, 2H), 7.67 (m, 2H), 8.06 (d, 1H), 8.11 (m, 2H); HRMS ($C_{30}H_{35}N_4O$) $[M+H]^+$: found *m/z* 467.2781, calcd 467.2805.

5.8.10.4. 4-(4-Methylpiperazine-1-carbonyl)-9-(2-(1*H*-indol-3-yl)-ethylamino)-(10*H*)-acridine (19d). Yield: 71.2%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 2.18 (br s, 1H), 2.32 (s, 3H), 2.43 (br s, 1H), 2.58 (br s, 1H), 2.69 (br s, 1H), 3.25 (br s, 4H), 3.88 (br s, 1H), 4.2 (br s, 3H), 5.28 (br s, 1H), 7.07 (br s, 1H), 7.15 (t, 1H), 7.25 (m, 3H), 7.42 (d, 1H), 7.61 (m, 2H), 7.64 (d, 1H), 7.97 (br m, 3H), 8.14 (br s, 1H); HRMS ($C_{29}H_{30}N_5O$) $[M+H]^+$: found *m*/*z* 464.2414, calcd 464.2445.

5.8.10.5. 4-(Morpholine-4-carbonyl)-9-(2-(1*H*-indol-3-yl)-ethylamino)-(10*H*)-acridine (19e). Yield: 72.0%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.23 (br s, 4H), 3.46 (br s, 1H), 3.87 (br m, 4H), 4.19 (br s, 3H), 5.29 (br s, 1H), 7.06 (br s, 1H), 7.16 (t, 1H), 7.25 (m, 3H), 7.41 (d, 1H), 7.62 (m, 2H), 7.65 (d, 1H), 7.95 (br m, 3H), 8.18 (br s, 1H); HRMS ($C_{28}H_{27}N_4O_2$) $[M+H]^+$: found *m*/*z* 451.2106, calcd 451.2128.

5.8.10.6. *N*,*N*-Diethyl-9-(2-(1*H*-indol-3-yl)-ethylamino)-(10*H*)-acridine-4-carboxamide (19f). Yield: 52.6%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.02 (br s, 3H), 1.41 (br s, 3H), 3.22 (br s, 2H), 3.75 (br m, 2H), 4.18 (br s, 2H), 5.22 (br s, 2H), 7.03 (br s, 1H), 7.14 (t, 1H), 7.25 (m, 3H), 7.40 (d, 1H), 7.56 (m, 2H), 7.65 (d, 1H), 7.96 (br m, 3H), 8.23 (br s, 1H); HRMS ($C_{28}H_{29}N_4O$) $[M+H]^+$: found *m*/*z* 437.2320, calcd 437.2336.

5.8.10.7. 3-(4-Methylpiperazine-1-carbonyl)-9-(1-benzylpiperidin-4-ylamino)-(10*H*)-acridine (19g). Yield: 40.1%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.76 (br m, 2H), 2.08 (br m, 4H), 2.34 (s, 3H), 2.41 (br s, 2H), 2.54 (br s, 2H), 2.88 (br s, 2H), 3.52 (s, 2H), 3.61 (br s, 2H), 3.88 (br s, 3H), 4.74 (br s, 1H), 7.26 (m, 1H), 7.31 (m, 4H), 7.46 (m, 2H), 7.73 (t, 1H), 8.07 (d, 1H), 8.08 (s, 1H), 8.12 (d, 1H), 8.14 (d, 1H); HRMS ($C_{31}H_{36}N_5O$) $[M+H]^+$: found *m*/*z* 494.2894, calcd 494.2914.

5.8.10.8. *N*,*N*-Diethyl-9-(1-benzylpiperidin-4-ylamino)-(10*H*)-acridine-3-carboxamide (19h). Yield: 10.4%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.17 (br s, 3H), 1.30 (br s, 3H), 1.75 (br m, 2H), 2.07 (br m, 4H), 2.88 (br m, 2H), 3.41 (br s, 2H), 3.52 (s, 2H), 3.63 (br s, 2H), 3.87 (br s, 1H), 4.72 (br s, 1H), 7.26 (m, 1H), 7.31 (m, 4H), 7.43 (m, 2H), 7.72 (t, 1H), 8.07 (m, 2H), 8.12 (m, 2H); HRMS ($C_{30}H_{35}N_4O$) $[M+H]^+$: found *m*/*z* 467.2779, calcd 467.2805.

5.8.10.9. 3-(4-Methylpiperazine-1-carbonyl)-9-(2-(1*H*-indol-3-yl)-ethylamino)-(10*H*)-acridine (19i). Yield: 29.6%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 2.33 (s, 3H), 2.39 (br s, 2H), 2.52 (br s, 2H), 3.25 (t, 2H), 3.56 (br s, 2H), 3.87 (br s, 2H), 4.23 (t, 2H), 7.09 (s, 1H), 7.17 (t, 1H), 7.26 (t, 1H), 7.31 (m, 2H), 7.43 (d, 1H), 7.67 (m, 2H), 7.92 (d, 1H), 7.96 (d, 1H), 8.02 (s, 1H), 8.06 (d, 1H), 8.21 (br s, 1H); HRMS ($C_{29}H_{30}N_5O$) $[M+H]^+$: found *m*/*z* 464.2414, calcd 464.2445.

5.8.10.10. *N*,*N*-Diethyl-9-(2-(1*H*-indol-3-yl)-ethylamino)-(10*H*)-acridine-3-carboxamide (19j). Yield: 45.5%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.14 (br s, 3H), 1.30 (br s, 3H), 3.32 (br s, 4H), 3.60 (br s, 2H), 4.29 (br s, 2H), 7.13 (m, 2H), 7.24 (m, 3H), 7.43 (d, 1H), 7.59 (t, 1H), 7.63 (d, 1H), 7.84 (br s, 1H), 7.94 (d, 1H), 8.06 (d, 1H), 8.10 (s, 1H), 8.48 (br s, 1H); HRMS ($C_{28}H_{29}N_4O$) $[M+H]^+$: found *m*/*z* 437.2308, calcd 437.2336.

5.8.10.11. 2-(4-Methylpiperazine-1-carbonyl)-9-(1-benzylpiperidin-4-ylamino)-(10*H*)-acridine (19k). Yield: 73.6%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.75 (br m, 2H), 2.08 (br m, 4H), 2.35 (s, 3H), 2.48 (br s, 4H), 2.88 (br s, 2H), 3.52 (s, 2H), 3.61 (br s, 2H), 3.88 (br m, 3H), 4.84 (br s, 1H), 7.26 (m, 1H), 7.31 (m, 4H), 7.45 (t, 1H), 7.69 (d, 1H), 7.73 (t, 1H), 8.07 (d, 1H), 8.13 (m, 3H); HRMS ($C_{31}H_{36}N_5O$) $[M+H]^+$: found *m*/*z* 494.2882, calcd 494.2914.

5.8.10.12. *N*,*N*-Diethyl-9-(1-benzylpiperidin-4-ylamino)-(10*H*)-acridine-2-carboxamide (19l). Yield: 50.7%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.23 (br s, 6H), 1.75 (br m, 2H), 2.07 (br m, 4H), 2.87 (br m, 2H), 3.41 (br s, 2H), 3.51 (s, 2H), 3.63 (br s, 2H), 3.88 (br s, 1H), 4.76 (br s, 1H), 7.26 (m, 1H), 7.30 (m, 4H), 7.44 (t, 1H), 7.68 (d, 1H), 7.74 (t, 1H), 8.07 (d, 1H), 8.12 (m, 2H), 8.22 (s, 1H); HRMS ($C_{30}H_{35}N_4O$) $[M+H]^+$: found *m*/*z* 467.2776, calcd 467.2805.

5.8.10.13. 2-(4-Methylpiperazine-1-carbonyl)-9-(2-(1*H*-indol-3-yl)-ethylamino)-(10*H*)-acridine (19m). Yield: 60.3%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 2.32 (s, 3H), 2.42 (br s, 4H), 3.23 (t, 2H), 3.49 (br s, 2H), 3.82 (br s, 2H), 4.23 (t, 2H), 7.14 (m, 2H), 7.24 (t, 1H), 7.30 (t, 1H), 7.42 (d, 1H), 7.64 (m, 3H), 7.94 (d, 1H), 8.04 (t, 2H), 8.07 (s, 1H), 8.43 (br s, 1H); HRMS ($C_{29}H_{30}N_5O$) $[M+H]^+$: found *m*/*z* 464.2415, calcd 464.2445.

5.8.10.14. *N*,*N*-Diethyl-9-(2-(1*H*-indol-3-yl)-ethylamino)-(10*H*)-acridine-2-carboxamide (19n). Yield: 82.8%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.11 (br s, 3H), 1.28 (br s, 3H), 3.22 (t, 2H), 3.26 (br s, 2H), 3.58 (br s, 2H), 4.24 (t, 2H), 7.11 (s, 1H), 7.15 (t, 1H), 7.24 (t, 1H), 7.30 (t, 1H), 7.42 (d, 1H), 7.64 (m, 3H), 7.93 (d, 1H), 7.97 (s, 1H), 8.01 (br s, 2H), 8.43 (br s, 1H); HRMS ($C_{28}H_{29}N_4O$) $[M+H]^+$: found *m*/*z* 437.2310, calcd 437.2336.

5.8.10.15. *N*,*N*-Diethyl-9-amino-(10*H*)-acridine-2-carboxamide (19o). Yield: 72.4%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.24 (br s, 6H), 3.38 (br s, 2H), 3.59 (br s, 2H), 5.70 (br s, 2H), 7.43 (t, 1H), 7.66 (d, 1H), 7.73 (t, 1H), 7.91 (d, 1H), 8.08 (m, 3H); HRMS ($C_{18}H_{20}N_3O$) $[M+H]^+$: found *m*/*z* 294.1588, calcd 294.1601.

5.8.10.16. *N*,*N*-Diethyl-9-(2-(dimethylamino)ethylamino)-(10*H*)-acridine-2-carboxamide (19p). Yield: 51.2%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.24 (br s, 6H), 2.37 (s, 3H), 2.62 (t, 2H), 3.49 (br s, 4H), 3.91 (t, 2H), 7.38 (t, 1H), 7.64 (d, 1H), 7.69 (t, 1H), 8.064 (d, 1H), 8.07 (d, 1H), 8.17 (d, 1H), 8.27 (s, 1H); HRMS ($C_{22}H_{29}N_4O$) $[M+H]^+$: found *m*/*z* 365.2307, calcd 365.2336.

5.8.10.17. *N*,*N*-Diethyl-9-(4-methylpiperazin-1-yl)-(10*H*)-acridine-2-carboxamide (19q). Yield: 43.1%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.18 (br s, 6H), 2.33 (s, 3H), 2.64 (t, 4H), 3.36 (br s, 4H), 3.61 (t, 4H), 7.56 (t,

1H), 7.70 (d, 1H), 7.79 (t, 1H), 8.09 (d, 1H), 8.10 (d, 1H), 8.25 (s, 1H), 8.37 (d, 1H); HRMS ($C_{23}H_{29}N_4O$) $[M+H]^+$: found *m*/*z* 377.2310, calcd 377.2336.

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