Identification of Compounds with Anti-West Nile Virus Activity

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The lack of antiviral compounds targeting flaviviruses represents a significant problem in the development of strategies for treating West Nile Virus (WNV), Dengue, and Yellow Fever infections. Using WNV high-throughput screening techniques developed in our laboratories, we report the identification of several small molecule anti-WNV compounds belonging to four different structural classes including pyrazolines, xanthanes, acridines, and quinolines. The initial set of "hits" was further refined using cell viability-cytotoxicity assays to two 1,3,5-triaryl pyrazoline compounds: 1-(4-chlorophenylacetyl)-5-(4-nitrophenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole and 1-benzoyl-5-(4-chlorophenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole. On the basis of their activity and favorable therapeutic indexes, these compounds were identified as viable leads and subjected to additional evaluation using an authentic viral titer reduction assay employing an epidemic strain of WNV. The compounds were further evaluated in a transient replicon reporting system to gain insight into the mechanism of action by identifying the step at which inhibition takes place during viral replication. The results indicate the pyrazolines inhibit RNA synthesis, pointing to viral RNA polymerase, RNA helicase, or other viral replication enzymes as potential targets. Progress was also made in understanding the structural requirements for activity by synthesizing a focused chemical library of substituted pyrazolines. Preliminary SAR data are presented that show the aryl-rings are required for activity against WNV. More importantly, the results indicate WNV activity is tolerant to aryl-substitutions paving the way for the design and development of much larger combinatorial libraries with varied physicochemical properties.

Introduction

Since the 1999 West Nile Virus (WNV) outbreak, there has been an increased interest in identifying compounds with anti-WNV activity.[1−4] The WNV is a member of the *Flavivirus* genus, which belongs to the *Flaviviдae* family. The *Flavivirus* genus includes a number of additional significant human pathogens such as yellow fever virus (YF), dengue virus (DEN), Japanese encephalitis virus (JE), Murray Valley encephalitis virus (MVE), St. Louis encephalitis virus (SLE), and tick-bourne encephalitis virus (TBE).[5−8] Viruses belonging to the *Flavivirus* genus are typically transmitted to vertebrates by mosquitoes or ticks and are responsible for severe morbidity and mortality in both humans and animals.[5−8]

Over the past 6 years, the WNV has spread to nearly every state in the continental U.S. and parts of Canada and Mexico.[9−11] During 2003 and 2004, the CDC reported more than 12,000 human cases in the United States, with at least 362 resulting in death.[10] Discovering compounds that can be used for prevention and treatment of WNV infections is becoming a general public health priority.[6,11] Currently, there are no viable drugs or vaccines available to treat or prevent WNV infection in humans.[3,14]

The WN virion consists of a single plus-sense RNA genome approximately 11 kb in length. The genomic RNA is composed of a 5′ and 3′ untranslated region (UTR) and a single open reading frame (ORF). The ORF encodes a total of 10 viral proteins: three structural (capsid, membrane, and envelope) and seven nonstructural, which are essential for viral replication.[5,12] Upon viral infection and release of genomic RNA, the plus-sense RNA can be translated into a single polyprotein that is then processed by viral and host proteases.[12] In addition, the plus-sense RNA is transcribed into minus-sense RNA, which subsequently acts as a template for the production of more plus-sense RNA.[5]

Recently, we have developed three separate systems for conducting high-throughput screening (HTS) assays: a cell line containing a persistently replicating subgenomic replicon, a full-length reporting virus, and packaged virus-like particles (VLPs) containing replicon RNA.[12,13] All three assays were validated using three known inhibitors of the WNV: ribavirin, glycyrrhizin, and mycophenolic acid.[13] Because each assay encompasses multiple yet distinct steps of the viral life cycle, these assays can be used to help elucidate the mechanism of action for potential inhibitors.[13] These new techniques offer an attractive alternative to traditional whole cell cytopathic assays, which are more time-consuming and offer little information about possible mechanisms of action. Because these assays are cell-based, it is also possible to investigate cellular uptake and chemical modification within the cell. Therefore, hits generated with these methods stand a greater chance of success in animal studies as compared to hits generated from target-based assays.[13]

In the current study, an initial library of 108 compounds was screened in the three HTS WNV assays. The library is a collection of compounds that have been synthesized in our laboratory over the last several years. The structural classes represented have a history of antimicrobial and antiviral activity and fall into six categories: pyrazolines, xanthones, xanthanes, acridones, acridines, and quinolines. Primary screening identified 24 "hits" from four of the structural classes. This set was further refined on the basis of cell cytotoxicity assays, resulting in the identification of two 1,3,5-triaryl pyrazoline lead compounds. Here, we describe the library synthesis and biological evaluation of the 24 potential WNV inhibitors and report preliminary structure−activity relationship (SAR) data for a second generation set of pyrazoline compounds. In addition, we discuss the

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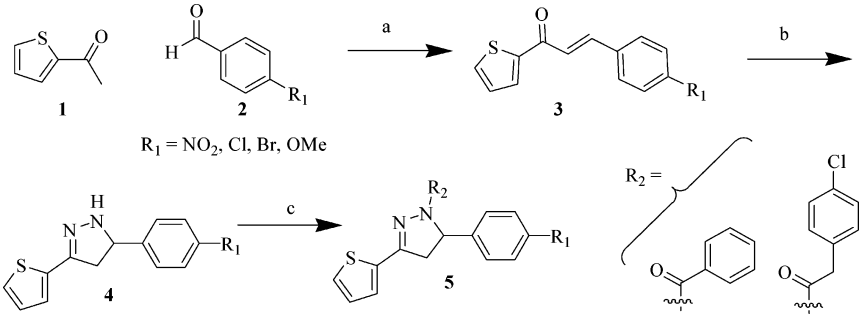
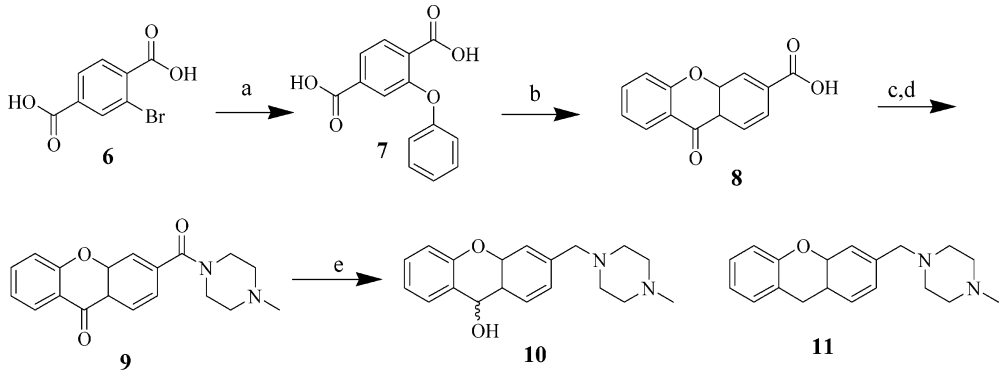
Scheme 1. Synthesis of Pyrazoline Compounds[a]



[a] Reagents and conditions: (a) NaOH, methanol or ethanol, room temperature; (b) $N_2H_4 \cdot H_2O$, ethanol, room temperature; (c) acid chloride, slight heat.

Scheme 2. Synthesis of Xanthane Compounds[a]



[a] Reagents and conditions: (a) phenol, Cu, CuI, pyridine, DBU, DMF, reflux; (b) H_2SO_4, 100 °C; (c) $SOCl_2$, reflux; (d) CH_2Cl_2, *N*-methylpiperazine; (e) DIBAL, THF.

validation of the prazoline hit **5a** and the initial steps toward the elucidation of the mechanism by which this compound inhibits WNV replication.

Chemistry

The synthesis of the pyrazolines began with a Claisen−Schmidt condensation between a methyl aryl ketone (**1**) and an aromatic aldehyde (**2**) affording the stable chalcone intermediate (**3**) as demonstrated in Scheme 1.[15] This reaction was conducted in the presence of a strong base, such as NaOH, in a minimal volume of methanol or ethanol. With little purification required, the chalcone was reacted with hydrazine in ethanol, which afforded the 3,5-diaryl pyrazoline (**4**).[16] Subsequent reaction with an acid chloride yielded the 1,3,5-trisubstituted pyrazoline (**5**).[16]

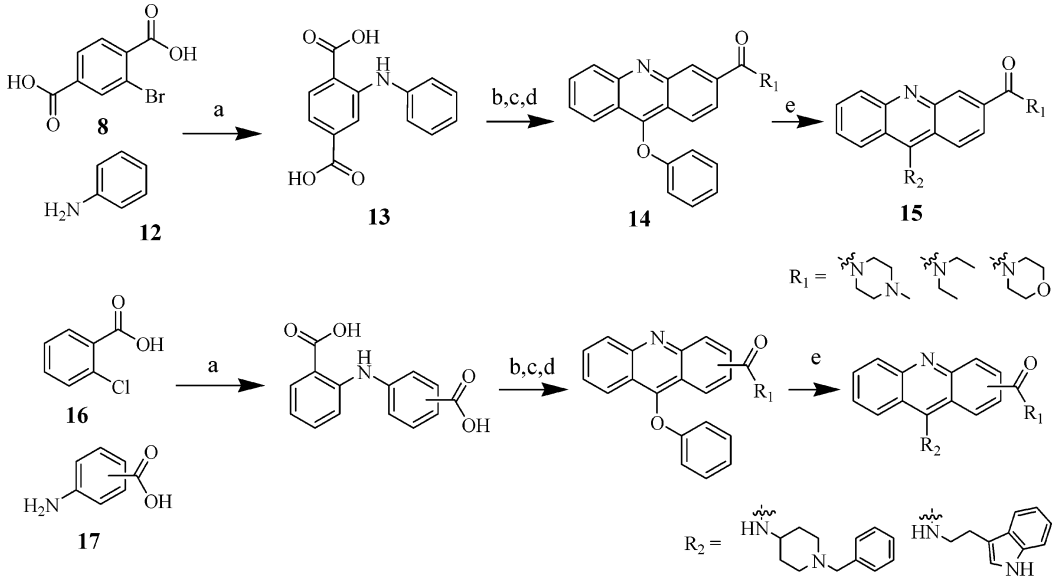
The xanthanes were synthesized from xanthones and hence followed the same synthetic path as the xanthones. As shown in Scheme 2, the first reaction was a modified Ullmann−Goldberg coupling between bromoterephthalic acid (**6**) and phenol, which was catalyzed by copper.[17,18] In this reaction, CuI and pyridine are cocatalysts responsible for maintaining the catalytic cycle of Cu^0, reducing the reaction time and increasing yields. The use of a nonnucleophilic 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 (**7**). Cyclization of **7** with concentrated H_2SO_4 at 100 °C yielded the substituted xanthone. Refluxing with $SOCl_2$ produced the acid chloride intermediate of compound **8**. Subsequent reaction of the acid chloride with *N*-methylpiperazine afforded the 3-carboxamide substituted xanthone (**9**). Reduction of the xanthone with a mild reducing agent, such as DIBAL in THF, produced a mixture of partially and fully reduced xanthanes (**10** and **11**, respectively). In this reaction, the fully reduced product was the predominant product. Attempts to produce the partially reduced hydroxyl product in greater portions by reaction modification were unsuccessful. Separation of **10** and **11** was achieved by silica chromatography.

The synthesis of acridines also began with a modified Ullmann−Goldberg coupling. In this case, however, aniline (**12**) was used in place of phenol, yielding the *N*-phenylanthranilic acid (**13**) as shown in Scheme 3.[17,18] Cyclization with $POCl_3$ generated the 9-chloroacridine intermediate in addition to producing an acid chloride of the second carboxylic acid.[19] This intermediate was then reacted with an amine, yielding the unstable 3-carboxamide-substituted-9-chloroacridine intermediate. Subsequent reaction with phenol and mild heating results in the stable 3-amide-9-phenoxyacridine (**14**). The final step entailed reacting the intermediate **14** with the desired amine, producing the substituted 9-aminoacridine (**15**).[20] To synthesize acridines substituted in the 2- and 4-positions, different reagents are required in the Ullmann−Goldberg coupling. Hence, the reaction between 2-chlorobenzoic acid (**16**) and the appropriate aminobenzoic acid (**17**) 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.

The synthesis of 4-aminoquinoline began with a $POCl_3$-mediated cyclodehydration reaction between anthranilic acid (**18**) and substituted acetophenone (**19**). This afforded the 2-phenly-3-chloroquinoline intermediate (**20**), as seen in Scheme 4.[21] Subsequent reaction of **20** with *N*-methylpiperazine under heating conditions yielded the 2-phenyl-4-aminoquinoline analogue (**21**).[22]

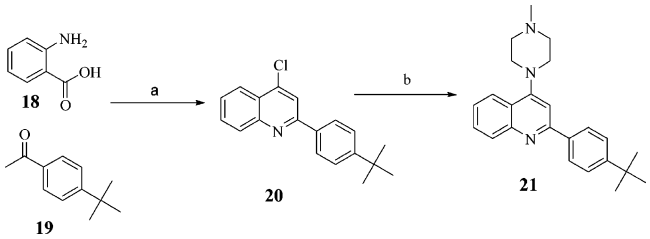
As shown in Scheme 5, the first step in the synthesis of 2-aminoquinoline involved the reaction of isatoic anhydride (**22**) with *N*,*O*-dimethylhydroxylamine (**23**), generating the weinreb amide (**24**).[23] Upon purification, amide **24** was reacted with the

Scheme 3. Synthesis of Acridine Compounds[a]



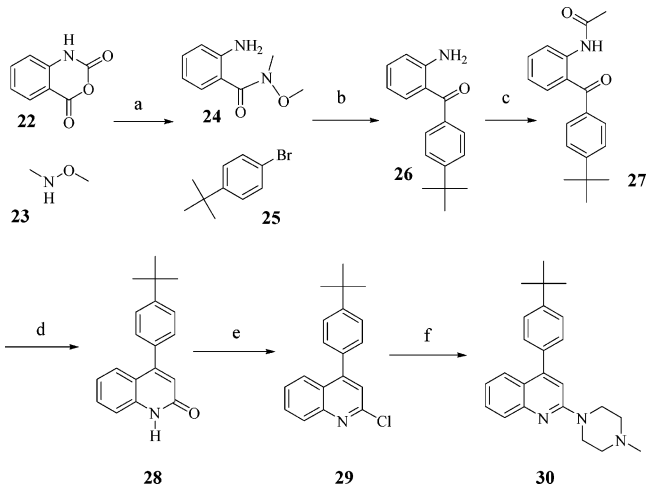
[a] Reagents and conditions: (a) Cu, CuI, pyridine, K_2CO_3, H_2O, reflux; (b) $POCl_3$, reflux; (c) amine, CH_2Cl_2, 0 °C; (d) phenol, 60 °C; (e) amine, 120 °C.

Scheme 4. Synthesis of 4-Aminoqiunoline (**21**)[a]



[a] Reagents and conditions: (a) $POCl_3$, reflux; (b) *N*-methylpiperazine, 120 °C.

Scheme 5. Synthesis of 2-Aminoquinoline[a]



[a] Reagents and conditions: (a) NEt_3, ethanol/H_2O, reflux; (b) *n*-BuLi, THF, −78 °C; (c) acetyl chloride, CH_2Cl_2, 0 °C; (d) NaOMe, ethanol, reflux; (e) $POCl_3$, reflux; (f) *N*-methylpiperazine, heating.

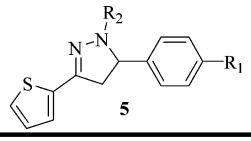
substituted bromobenzene (**25**) in the presence of *n*-butyllithium, generating the benzophenone intermediate (**26**).[23] At this point, the amino group was actylated with acetyl chloride, generating **27**.[24] Lactamization of **27** was then achieved by treating with sodium methoxide in ethanol under reflux conditions.[25] Treatment of **28** with $POCl_3$ produced the 2-chloroquinoline (**29**), which was then reacted with *N*-methylpiperazine, yielding the 2-amino-4-phenylquinoline analogue (**30**).

Biology

Three cell-based high-throughput screening (HTS) assays were used to identify and characterize inhibitors of WNV.[13] The first uses a reporting replicon-containing cell line. As compared to the wild-type genomic RNA, the reporting replicon contains a deletion of viral structural genes. In addition, a *Renilla* luciferase gene (Rluc) and a Neomycin resistance gene (Neo) were engineered into the replicon, resulting in a reporting replicon (Rluc-Neo-Rep). The cell lines containing the replicating Rluc-Neo-Rep RNAs can be used to screen for inhibitors of viral replication (including viral translation and RNA synthesis), but not viral assembly and entry. The second applies a virus-like particle (VLP) infection assay. The VLPs were prepared by expressing WNV structural proteins in cells containing the Rluc-Neo-Rep. The structural proteins could package the Rluc-Neo-Rep RNAs to form infectious VLPs. Infection of naive cells with such VLPs allows screening inhibitors of viral entry and replication, but not viral assembly (because the replicon contains a deletion of viral structural proteins). The third is a full-length reporting WNV assay. The full-length reporting WNV contains a Rluc reporter (Rluc-FL-WNV) and can be used to screen inhibitors against all steps of a viral life cycle, including viral entry, replication, and virion assembly. Because luciferase has been engineered into each of the assays described above, inhibition is measured through suppression of luciferase signals. A more detailed description of the three HTS assays has been recently reported.[13]

All compounds were initially subjected to two primary screens: the reporting-replicon (Rluc-Neo-Rep) BHK cell line assay and the full-length reporting (Rluc-FL-WNV) infection assay in Vero cells. This combination can be applied to delineate inhibition of viral translation/RNA synthesis from entry and assembly processes. Each compound was preliminarily screened at 30 μM at a 1% DMSO final concentration. Potential WNV inhibitors showing greater than 50% inhibition of Rluc activity were then analyzed for cytotoxicity using an MTT assay.[27] Cytotoxicity of the compounds was determined in both Vero and BHK cells. Hits from the primary screen were then screened in the replicon-containing VLP infection assay.

Table 1. WNV Screening Results for Pyrazoline-Based Compounds



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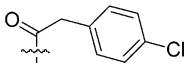
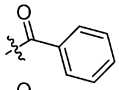
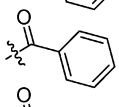
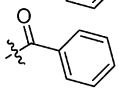
			Rluc-Neo-Rep in BHK cells			Rluc-FL-WNV in Vero cells			VLP[a]	Rluc-Neo-Rep[b]
Compound	R_1	R_2	EC_{50} μM	CC_{50} μM	TI[a]	EC_{50} μM	CC_{50} μM	TI[b]	EC_{50} μM	EC_{50} μM
5a	NO_2		11	>300	>27	25	>300	>12	8	7
5b	Cl		17	>300	>18	23	>300	>13	15	15
5c	Br		25	>300	>12	>>30	ND	ND	ND	ND
5d	OMe		63	>300	>4.8	>>30	ND	ND	ND	ND
Ribavirin			18			30			1.1	119
Mycophenolic Acid			1.4			0.04			0.04	0.08

[a] BHK-21 cells. [b] Vero cells, ND = not determined because initial screen was ≫30 mM.

Table 2. WNV Screening Results for Xanthane-Based Compounds

	Rluc-Neo-Rep in BHK cells			Rluc-FL-WNV in Vero cells			VLP[a]
compound	EC_{50} μM	CC_{50} μM	TI[a]	EC_{50} μM	CC_{50} μM	TI[b]	EC_{50} μM
10	13.5	325	24	10	50	5	4
11	>30	200	<6.7	>30	90	<3	28

[a] BHK-21 cells. [b] Vero cells.

Results

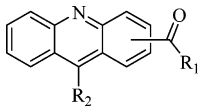
Pyrazolines. The initial screen contained 15 compounds belonging to the pyrazoline structural class. From this small set, two derivatives showed significant activity in all three HTS assays, as shown in Table 1. Activity across all three assays strongly suggests these compounds are most likely disrupting viral replication (see Biology section). More importantly, the pyrazolines **5a** and **5b** show low toxicity in both BHK and Vero cell lines. In comparison to the known inhibitor ribavirin, it was observed that both of these pyrazolines showed greater activity. Given this finding, and the favorable therapeutic index of the pyrazolines, **5a** and **5b** have been identified as WNV lead compounds. These compounds are racemic mixtures, however, indicating that gains in activity may be afforded through chiral separation. In addition, the finding that several other 1,3,5-triaryl pyrazolines (compounds **5c** and **5d** in Table 1) do not exhibit significant activity suggests that SAR studies might be valuable in refining lead compounds **5a** and **5b**. Although comprehensive SAR is beyond the scope of this report, preliminary SAR data are reported below.

Xanthanes and Xanthones. The library screened contained eight xanthanes and 10 of the parent xanthones. From these two classes, there were only two compounds that exhibited anti-WNV activity. Coincidentally, they are both products from the same reaction: the fully reduced xanthane and the partially reduced 9-hydroxyxanthane. Further, the 9-hydroxy compound (**10**) is racemic and was the only 9-hydroxy compound submitted to the screen. The results reported in Table 2 show compound **10** as being more active than the fully reduced analogue (**11**) and ribavirin. As was the case with the pyrazoline derivatives, compound **10** exhibited activity over all three HTS assays, once again suggesting inhibition of the replication process. The final observation to note is the difference in toxicity between BHK and Vero cells. It appears that compound **10** is approximately 6.5 times more toxic to Vero cells versus BHK cells. Because compound **10** is racemic and the toxicity may simply be due to one of the enantiomers, it is possible that chiral separation could result in a compound with greater activity and reduced toxicity.

Acridines and Acridones. The majority of the compounds submitted to the screen belong to the acridine class or the related acridone class consisting of 29 and 40 analogues, respectively. From these two classes, 18 hits were discovered, all belonging to the acridine class. Structurally speaking, the primary differences between these two classes are the substitution at the 9 position (R_2) of the acridine and the aromaticity of the middle ring. In the case of the acridone compounds, the middle ring is not aromatized and bears no substitution, except a ketone at the 9 position. From the results shown in Table 3, it is clear that little to no SAR can be drawn, despite having a comprehensive set of compounds. The lack of substantial effect by various substitutions may indicate that the mechanism whereby these compounds display anti-WNV activity is not specific and accommodates various functionalities. It is possible that aromaticity of the acridine core structure plays a major role, which would partly explain the lack of activity with similar acridones. Once again, because the activity is seen in all three assays, it strongly suggests the acridines target the replication process as well. Although these analogues demonstrate reasonable activities as compared to ribavirin, they also appear to have greater toxicities, particularly in the Vero cells.

Quinolines. The quinoline class of compounds, six in all, represents the smallest set of compounds in our screening.

Table 3. WNV Screening Results for Acridine-Based Compounds



				Rluc-Neo-Rep in BHK cells			Rluc-FL-WNV in Vero cells			VLP[a]
compound	ring position	R_1	R_2	EC_{50} μM	CC_{50} μM	TI[a]	EC_{50} μM	CC_{50} μM	TI[b]	EC_{50} μM
14a	4	*N*-methy1-piperazinyl	phenoxy	20	150	7.5	20	50	2.5	20
14c	4	morpholino	phenoxy	>30	155	<5.1	22	45	2	>30
15a	4	*N*-methy1-piperazinyl	4-amino-*N*-benzylpiperdinyl	15	300	20	22	80	3.6	20
15b	4	diethylamino	4-amino-*N*-benzylpiperdinyl	8	350	44	22	75	3.4	20
15c	4	morpholino	4-amino-*N*-benzylpiperdinyl	20	300	15	>30	90	<3	20
15d	4	*N*-methy1-piperazinyl	tryptamino	10.5	150	14	18	50	2.8	17
15e	4	diethylamino	tryptamino	9.2	150	16	20	50	2.5	9
15f	4	morpholino	tryptamino	20	250	13	>30	60	<2	23
14d	3	*N*-methy1-piperazinyl	phenoxy	28	100	3.6	28	40	1.4	18
15g	3	*N*-methy1-piperazinyl	4-amino-*N*-benzylpiperdinyl	15.5	150	9.7	30	80	2.7	8
15h	3	diethylamino	4-amino-*N*-benzylpiperdinyl	12.5	150	12	20	55	2.7	6
15i	3	*N*-methy1-piperazinyl	tryptamino	13.7	150	11	>30	45	<1.5	10
15j	3	diethylamino	tryptamino	7	125	18	25	45	1.8	7
14f	2	*N*-methy1-piperazinyl	phenoxy	21	100	4.7	20	80	4	20
15k	2	*N*-methy1-piperazinyl	4-amino-*N*-benzylpiperdinyl	28	100	3.6	25	80	3.2	25
15l	2	diethylamino	4-amino-*N*-benzylpiperdinyl	22	120	5.4	20	50	2.5	22
15m	2	*N*-methy1-piperazinyl	tryptamino	28	170	6.1	25	65	2.6	27
15n	2	diethylamino	tryptamino	25	110	4.4	15	75	5	20

[a] BHK-21 cells. [b] Vero cells.

Table 4. WNV Screening Results for Quinoline-Based Compounds

	Rluc-Neo-Rep in BHK cells			Rluc-FL-WNV in Vero cells			VLP[a]
compound	EC_{50} μM	CC_{50} μM	TI[a]	EC_{50} μM	CC_{50} μM	TI[b]	EC_{50} μM
21	14.5	50	3.4	15	20	1.3	8
30	17	110	6.5	30	25	0.8	20

[a] BHK-21 cells. [b] Vero cells.

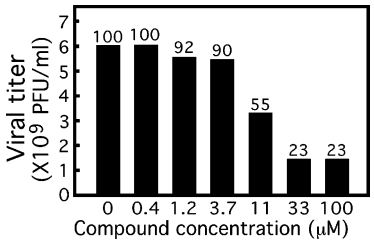


Figure 1. Inhibition of an epidemic strain of WNV by compound **5a**. Percent inhibition of viral titer at each concentration of compound is reported on the top of the columns.

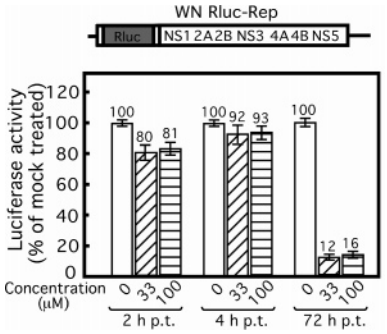


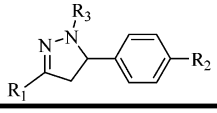
Figure 2. A WNV reporting replicon containing a luciferase reporter (fused in-frame with the ORF; Rluc-Rep) was used to measure the effects of **5a** on viral translation and RNA synthesis at indicated time points post-transfection (p.t.). Values above each data point indicate the percentage of luciferase signals from the compound-treated transfection as compared to that from the mock-treated transfection. Average results of two to three experiments are shown.

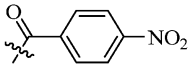
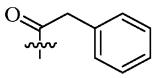
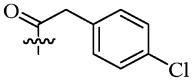
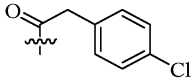
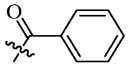
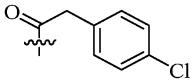
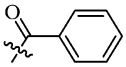
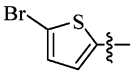
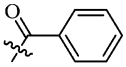
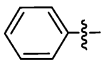
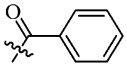
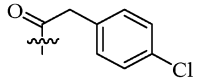
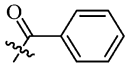
Similar to the acridine derivatives, the quinoline compounds demonstrated activity in all three HTS assays, as well as significant toxicities as shown in Table 4. This is not surprising, considering some of the similarities between the two classes. In comparing compounds **21** and **30**, it should be pointed out that the only difference is the exchange of the substitutions at the 2- and 4-positions. This modification does not appear to have much effect on activity, which may suggest that there is little structural specificity for these compounds. As with the acridines, the usefulness of these compounds is limited by their low TI ratios.

Verification of Anti-WNV Activity of 5a. To validate the anti-WNV potency of **5a**, the compound was additionally tested in an authentic viral titer reduction assay.[36] In this assay, Vero cells were first infected with an epidemic strain of WN virus followed immediately by treatment with compound. Viral yields were then assayed 42 h post infection. The results shown in Figure 1 indicate that **5a** inhibited viral titer in a dose response manner with an EC_{50} value estimated to be 15 μM, which is in good agreement with the three HTS assay results reported in Table 1.

Mechanism of Action of 5a. The results taken from the primary screens (Table 1) indicate that **5a** is active in both the Rluc-VLP and the Rluc-Neo assays, suggesting the compound functions by either inhibiting viral translation or RNA synthesis. To further evaluate the mechanism of action, **5a** was assayed using a transient WNV replicon system as previously reported.[36] This assay is able to distinguish between compound-mediated inhibition of viral translation and inhibition of RNA synthesis.[37] BHK cells transfected with WN RlucRep RNA were incubated with compound (at 33 and 100 μM) and assayed for luciferase

Table 5. WNV Screening Results for Trisubstituted Pyrazolines Analogues of **5a** and **5b**



Compound	R_1	R_2	R_3	Rluc-Neo-Rep in BHK cells		
				EC_{50} μM	CC_{50} μM	TI
31		Cl		225	>300	>1.3
32		Cl		20	65	3.3
33		Cl		19	>300	>16
34		Cl		60	90	1.5
35		Br		19	>300	>16
36		F		59	100	1.7
37		OMe		30	>300	>10
38		NO_2		200	>300	>1.5
39		Cl		17	>300	>18
40		Cl		20	250	12.5
41		Br		20	>300	>15
42		Cl		57	65	1.1
43		Cl		27	60	2.2

activities at 2, 4, and 72 h post-transfection. The 2 and 4 h time points represent inhibition of viral translation, whereas the 72 h time point represents inhibition of RNA synthesis. As shown in Figure 2, there was no significant suppression of luciferase activity at the 2 and 4 h time points, therefore compound **5a** does not inhibit viral translation. In contrast, the luciferase activity at the 72 h time point was suppressed by approximately 85%, indicating that **5a** inhibits WNV through suppression of viral RNA synthesis. These results are also supported by more traditional time-of-addition assays, which are reported elsewhere for compound **5b**.[36]

Preliminary SAR. To provide insight to potential SAR and to further evaluate the trisubstituted pyrazolines for development as WNV inhibitors, a preliminary set of analogues based on **5a** and **5b** has been synthesized. The results from this exploratory set of compounds are reported in Table 5. Even with this small focused set of compounds, SAR trends are beginning to emerge. A decrease of activity was observed when R_1 and R_3 of the triaryl pyrazoline were substituted with alkyls (**34**, **42**, and **43**), suggesting 1,3,5-triaryl substitutions are key to antiviral activity. The antiviral activity was also reduced for the R_3-4-nitrobenzoyl substitution (**31**). While the data presented here are limited (and should be applied with some caution), a general trend is evident for the R_3-4-chlorophenylacetyl substituted compounds (**33**, **35**, **37**, **41**, and also **5a** from Table 1), which all tend to show similar or improved activity as compared to their R_3-benzoyl analogues. The activity of the bromo-substituted derivative (**39**) is particularly interesting and suggests that bulky as well as polar substitutions to the thiophene ring can be tolerated.

Discussion

The lack of a practical therapeutic agent for the treatment of WNV is a growing concern. Current inhibitors of WNV are not viable candidates for various reasons. In particular, the broad spectrum anti-viral nucleoside, ribavirin, is limited by the dose required and toxicities associated with size of the dose.[3] Therefore, the discovery of new compounds with anti-WNV activity is of utmost importance. Because of their reasonable activity and favorable therapeutic index, the pyrazolines, **5a** and **5b**, exhibit the greatest promise as anti-WNV agents. The status of the pyrazoline **5a** as a genuine anti-WNV lead was determined by conducting a viral titer reduction assay. The close

agreement between the EC_{50}'s for the HTS assays and the WNV titer reduction assay is conclusive, indicating that **5a** is a valid WNV antiviral.

Since inhibition was seen in all three HTS assays, the mechanism of action can be characterized as a replication inhibitor rather than a viral entry or an assembly inhibitor. The mechanism of action for **5a** was further evaluated using the transient reporting replicon system, which differentiates between viral translation and RNA synthesis.[11,13,36] The results for **5a** indicated that the pyrazoline compound significantly repressed WNV RNA synthesis without dramatic suppression of viral translation. Inhibiting replication in this manner could entail the disruption of RNA synthesis by inhibiting viral RNA polymerase, RNA helicase, or other encoded viral replication enzymes. Target assays using the multifunctional WNV proteins NS3 and NS5 or mapping of mutant genes in resistant virus would have to be used to offer a more specific determination of the mechanism.[13]

In conducting these HTS assays, cell line selection may show significant influence on assay sensitivity and hence is a legitimate concern. For example, cellular functions within the cell are not entirely the same in Vero and BHK-21 cells. Ribavirin, in particular, requires phosphorylation inside the cell, which is known to be less efficient in Vero cells than in MA-104 cells.[1] In validating the three HTS assays used here, it was similarly found that ribavirin demonstrated significantly reduced activity in Vero versus BHK cells.[13] In contrast, mycophenolic acid, which does not require modification prior to its inhibition of viral replication through depleting the intracellular GTP pool, showed comparable activities in both Vero and BHK cell lines.[13] Because cell line selection could possibly affect assay sensitivity, we used both BHK-21 cells and Vero cells in our two primary screens. One primary screen was performed in BHK-21 cells containing the Rluc-Neo-Rep. The other screening was conducted in Vero cells infected with the full-length Rluc-WNV. To verify that the pyrazolines were not experiencing cell line dependence, they were also screened in Rluc-Neo-Rep containing Vero cells. Results here indicate that the pyrazolines, unlike ribavirin, do not show a cell line dependence (Table 1).

The class of compounds that produced the most hits in the three screens was the acridines. However, these compounds also possess the greatest toxicity, which is believed to be due to the intercalative nature of acridines into DNA. It is well known that planar polyaromatic compounds, such as acridines, are good intercalators and have been widely investigated as antitumor agents.[28,29] An example of this is the acridine, amsacrine, which is a known intercalator with potent topoisomerase II inhibition and is approved for use as an anti-leukemia drug.[28] Upon intercalation, these compounds can feasibly affect any cellular enzyme that processes DNA.[30,31] Such enzymes would include polymerases, helicases, topoisomerases, and gyrases.[31,32] It is also possible for these compounds to intercalate in dsRNA stem loop regions, thus preventing enzyme recognition or processing.[33−35] With this knowledge, the presumed mechanism of action for these compounds in the WNV assays is most likely viral replication. Considering that the acridine derivatives are demonstrating WNV inhibition and cytotoxicity via seemingly the same mechanism, it is unlikely that structural modification of the acridine derivatives will yield compounds with more favorable therapeutic indices. Presently, we are under the assumption that the quinolines, which bear several structural similarities with the acridines, will not benefit from structural modification as well.

Conclusion

In summary, the primary screening of 108 compounds has led to the discovery of two 1,3,5-triaryl pyrazoline compounds with promising therapeutic indices and activity against WNV. The leads were also applied to the design and development of a focused chemical library to further evaluate the initial "hits" and gain insight into the requirements for activity. Although the second generation library reported here is somewhat limited, the results indicate that additional combinatorial chemistry/SAR studies may be useful in refining the activity of these compounds against WNV and for identifying compounds with improved physicochemical properties (i.e., ADME). In this regard, the pyrazolines offer significant advantages, due in part by the ease of synthesis and amenability of chalcone chemistry to combinatorial library design.[38] Steps were also taken to determine the molecular target for these compounds using a transient reporting replicon assay. While the results point to a mechanism that involves inhibition of RNA synthesis, the details of this mechanism are not yet known. It is unlikely, however, that the pyrazolines function through nonspecific binding, such as intercalation, since the structures do not adopt planar conformations.

Finally, it is important to point out that this work has shown that several heterocycles related to well-known drug compounds (such as amsacrine and chloroquine) are potential inhibitors of WNV. We did, in fact, screen a short list of known acridine- and quinoline-based drugs and found that amsacrine and acriflavin were both active below 3.3 μM. While compounds of this type suffer from high toxicity, the results are quite interesting and will undoubtedly spark interest in evaluating heterocycles of this type in WNV and other flavivirus assays. In this regard, the xanthanes may be of particular interest. Whereas the acridines are thought to function through intercalation (leading to inherent toxicities), the mechanism of action of xanthanes has not been established, providing some hope that toxicity can be factored out of the equation.

Experimental Section

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 via a Millipore filtration system. Column chromatography was conducted using silica gel 60 (40−63 μm), 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. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Beckman Coulter 125S System Gold using an Agilent Zorbax Eclipse XDB-C8 3.5 μM 3.0 × 150 mm column monitoring UV at 254λ on a 166 detector. Method A was a gradient method that ran for 14 min at a flow rate of 0.5 mL/min. Over the first 8 min, the percent ACN/water was increased from 60% to 100%. Over the next 4 min, the gradient was decreased back to 60% ACN/water and then held for 2 min. Method B was an isocratic method using 70% MeOH/water and a flow rate of 0.4 mL/min. The run duration was 20 min. Results were analyzed using the 32 Karat software package.

General Procedure for the Chalcone Intermediate (3). Aromatic acetyl (**1**) (5 mmol) and aromatic aldehyde (**2**) (5 mmol) were dispersed in either ethanol or methanol (∼3 mL). Next, two NaOH pellets (∼0.24 g) were added, and the reaction was stirred while slightly heating until starting materials were completely dissolved.

Note that heating was not always necessary. Once dissolved, the reaction was removed from heat and stirred overnight. The product precipitated out and was filtered. Washing with cold methanol and water affords the chalcone, which was used without further purification. The product is a mixture of cis and trans alkenes, which convolutes the NMR spectra.

3-(4-Nitrophenyl)-1-thiophen-2-yl-propenone (3a). Yield = 94.8%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.22 (dd, J = 3.9 and 4.9 Hz, 1H), 7.52 (d, 1H), 7.75 (dd, J = 1.0 and 4.9 Hz, 1H), 7.79 (d, J = 8.8, 2H), 7.82 (d, 1H), 7.90 (dd, J = 0.98 and 3.8 Hz, 1H), 8.29 (d, J = 8.8, 2H).

3-(4-Chlorophenyl)-1-thiophen-2-yl-propenone (3b). Yield = 80.1%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.20 (dd, J = 3.8 and 4.9 Hz, 1H), 7.38 (m, 3H), 7.59 (d, 2H), 7.71 (dd, J = 1.1 and 4.9 Hz, 1H), 7.81 (d, 1H), 7.88 (dd, J = 1.1 and 3.8 Hz, 1H).

General Procedure for the 3,5-Diaryl Pyrazoline Intermediate (4). The chalcone intermediate (**3**) (5 mmol) was stirred in ethanol (~5 mL), and hydrazine monohydrate (12.5 mmol) was added. The reaction will go into solution and fall back out. Some light heating may be required for the reaction to go into solution. The resulting product was then passed through a fritted filter and washed with a cold 50/50 mix of methanol/water, quickly rinsed with methanol, and then briefly air-dried. The product sometimes undergoes decomposition and was used in the next reaction as soon as the product appeared dry without further purification.

General Procedure for 1,3,5-Trisubstituted Pyrazolines (5a−5d). The 3,5 diaryl pyrazolines (~5 mmol) were placed in a medium-sized vial, and excess amounts of the desired acid chloride were added. The reaction was then heated as HCl gas evolved. After approximately 5 min, the reaction was cooled and methanol (~2 mL) was added. The reaction was heated to dissolve any solid and cooled. The product that crystallized out was filtered and recrystallized in methanol to yield the 1,3,5-trisubstituted pyrazoline.

1-(4-Chlorophenylacetyl)-5-(4-nitrophenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole (5a). Yield = 60.8%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.12 (dd, J = 5.1 and 17.6 Hz, 1H), 3.82 (dd, J = 11.9 and 17.6 Hz, 1H), 4.04 (s, 2H), 5.60 (dd, J = 5.1 and 11.9 Hz, 1H), 7.09 (dd, J = 3.7 and 5.0 Hz, 1H), 7.22 (dd, J = 1.0 and 3.7 Hz, 1H), 7.29 (m, 6H), 7.49 (dd, J = 1.0 and 5.0 Hz, 1H), 8.15 (d, J = 8.8, 2H); HRMS ($C_{21}H_{17}ClN_3O_3S$) $[M + H]^+$: found m/z 426.0644, calcd 426.0673; RP-HPLC method A, t_R = 5.12 min (>99%); method B, t_R = 5.62 min (>99%).

1-Benzoyl-5-(4-chlorophenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole (5b). Yield = 47.3%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.16 (dd, J = 5.0 and 17.5 Hz, 1H), 3.81 (dd, J = 11.8 and 17.5 Hz, 1H), 5.79 (dd, J = 5.0 and 11.8 Hz, 1H), 7.07 (dd, J = 3.7 and 5.1 Hz, 1H), 7.24 (dd, J = 1.1 and 3.7 Hz, 1H), 7.29 (m, 7H), 8.01 (d, J = 7.0, 2H); HRMS ($C_{20}H_{16}ClN_2OS$) $[M + H]^+$: found m/z 367.0643, calcd 367.0666; RP-HPLC method A, t_R = 4.82 min (>99%); method B, t_R = 5.82 min (>99%).

1-Benzoyl-5-(4-bromophenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole (5c). HRMS ($C_{2O}H_{16}BrN_2OS$) $[M + H]^+$: found m/z 411.0030, calcd 411.0172.

1-Benzoyl-5-(4-methoxyphenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole (5d). HRMS ($C_{21}H_{19}N_2O_2S$) $[M + H]^+$: found m/z 363.1060, calcd 363.1172.

2-Phenoxyterephthalic Acid (7). Bromotherethphalic acid (**8**) (12.4 mmol) was added to 100 mL of 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 was heated to reflux and monitored via TLC. After 2 h, TLC showed that all of the bromotherethphalic acid was consumed. The reaction 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).

9-Oxo-9H-xanthene-3-carboxylic Acid (8). 2-Phenoxy-terephthalic acid (**7**) (10.6 mmol) was added to 100 mL of H_2SO_4 and heated to 100 °C while being stirred for 3 h. The starting material dissolved upon heating. The reaction was cooled and poured over ice (300 mL), producing a gray solid that 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).

3-(4-Methylpiperazine-1-carbonyl)-xanthen-9-one (9). Thionyl chloride (~25 mL) was added to a flask containing 9-oxo-9H-xanthene-3-carboxylic acid (**8**) (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 was allowed to reach room temperature under continued stirring for about 1 h. An additional 30 mL of CH_2Cl_2 was added, and the reaction 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 (s br, 2H), 2.52 (s br, 2H), 3.43 (s br, 2H), 3.83 (s br, 2H), 7.39 (m, 2H), 7.51 (m, 2H), 7.74 (t, 1H), 8.32 (d, 1H), 8.37 (d, 1H).

Synthesis of 3-(4-Methylpiperazin-1-ylmethyl)-9H-xanthen-9-ol (10) and 1-Methyl-4-(9H-xanthen-3-ylmethyl)-piperazine (11). 3-(4-Methyl-piperazine-1-carbonyl)-xanthen-9-one (**9**) (0.775 mmol) was dissolved in dry tetrahydrofuran (THF) (~25 mL) and cooled under argon to 0 °C on an ice bath. Next, diisobutylaluminum hydride (DIBAL) (4.65 mmol, 4.65 mL of 1.0 M solution in toluene) was added. Use of fewer equivalents only resulted in more unreduced starting material. The reaction was allowed to warm to room temperature and stirred overnight (~14 h). The reaction was then quenched with distilled water and extracted with diethyl ether (4×), dried with $MgSO_4$, and concentrated. TLC showed two spots when eluted with a 9:1 mix of CH_2Cl_2:MeOH. The product was purified by silica chromatography, yielding the partially reduced 3-(4-methyl-piperazin-1-ylmethyl)-9H-xanthen-9-ol (**10**) and the fully reduced 1-methyl-4-(9H-xanthen-3-ylmethyl)-piperazine (**11**). **10** yield ~11%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.26 (s, 3H), 2.45 (s br, 8H), 2.97 (s, 1H), 3.52 (s, 2H), 5.76 (s, 1H), 7.15 (m, 4H), 7.36 (m, 1H), 7.49 (m, 1H), 7.59 (m, 1H); HRMS ($C_{21}H_{19}N_2O_2S$) $[M + H]^+$: found m/z 367.0424, calcd 367.0666. **11** yield ~53%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.29 (s, 3H), 2.48 (s br, 8H), 3.48 (s, 2H), 4.03 (s, 2H), 7.00 (m, 4H), 7.10 (d, 1H), 7.17 (m, 2H); HRMS ($C_{19}H_{23}N_2O$) $[M + H]^+$: found m/z 295.1795, calcd 295.1805.

General Procedure for Phenylamino-terephthalic Acid (13a−c). 2-Phenylamino-terephthalic Acid (13a). Potassium carbonate (30.6 mmol) was first dissolved in 100 mL of distilled water. Next, 2-bromotherethphalic acid (**8**) (20.4 mmol) was added followed by aniline (**12**) (40.8 mmol), pyridine (0.3 mL), copper(0) (0.1 g), and copper(I) iodide (0.1 g). The reaction was stirred under reflux for 4 h. Once the reaction 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 filtrated 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).

***N*-(2-Carboxyphenyl)anthranilic Acid (13b).** Yield ~68%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.93 (m, 2H), 7.44 (m, 4H), 7.88 (m, 2H).

***N*-(4-Carboxyphenyl)anthranilic Acid (13c).** 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).

General Procedure for Intermediates 14a−g. (4-Methylpiperazin-1-yl)-(9-phenoxyacridin-4-yl)-methanone (14a). 2-Phenylamino-terephthalic acid (**13**) (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 resulting residue was then dissolved in CH_2Cl_2 (~50 mL) and cooled to 0 °C on an ice bath. Then excess amounts of desired amine (for example, *N*-methylpiperazine) (4 mL) were added dropwise while stirring. The reaction was allowed to warm to room temperature and then slightly heated while stirring for approximately 1 h. The reaction was then condensed and left on vacuum to remove residual amounts of amine. After 2 days on the pump, 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 was then cooled and diluted with 200 mL of CH_2Cl_2. The mixture was then washed with 30% w/w NaOH in water (150 mL × 2) to remove phenol, washed with brine (150 mL), dried over $MgSO_4$, and concentrated. TLC 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%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.22 (m br, 1H), 2.35 (s, 3H), 2.44 (m br, 1H), 2.60 (m br, 1H), 2.74 (m br, 1H), 3.24 (m br, 2H) 3.96 (m br, 1H), 4.16 (m br, 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.

9-Phenoxy-acridine-4-carboxylic Acid Diethylamide (14b). Yield = 41.5%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.96 (s br, 3H), 1.24 (s br, 3H), 3.21 (s br, 2H), 3.53 (s br, 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).

Morpholin-4-yl-(9-phenoxyacridin-4-yl)-methanone (14c). Yield = 45.1%; ^{1}H 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.

(4-Methylpiperazin-1-yl)-(9-phenoxy-acridin-3-yl)-methanone (14d). Yield = 47.5%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.34 (s, 3H), 2.41 (s br, 2H), 2.55 (s br, 2H), 3.58 (s br, 2H), 3.89 (s br, 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.

9-Phenoxyacridine-3-carboxylic Acid Diethylamide (14e). Yield = 55.5%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.97 (s br, 3H), 1.26 (s br, 3H), 3.23 (s br, 2H), 3.54 (s br, 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).

(4-Methylpiperazin-1-yl)-(9-phenoxy-acridin-2-yl)-methanone (14f). Yield = 41.3%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.18 (s br, 2H), 2.30 (s, 3H), 2.46 (s br, 2H), 3.36 (s br, 2H), 3.79 (s br, 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.

9-Phenoxy-acridine-2-carboxylic Acid Diethylamide (14g). Yield = 42.7%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.96 (s br, 3H), 1.24 (s br, 3H), 3.21 (s br, 2H), 3.53 (s br, 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).

General Procedure for Compounds 15a−n. [9-(1-Benzylpiperidin-4-ylamino)-acridin-4-yl]-(4-methylpiperazin-1-yl)-methanone (15a). (4-Methyl-piperazin-1-yl)-(9-phenoxy-acridin-4-yl)-methanone (**14a**) (10 mmol) was dissolved in 2 mL of 4-amino-*N*-benzylpiperidine and heated to 120 °C (reflux) for 15 min. The reaction 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 collect and concentrated. The yellow product was dissolved in EtOAc and precipitated out with petroleum ether or hexanes. Yield = 74.1%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.74 (s br, 2H), 2.06 (m br, 4H), 2.18 (s br, 1H), 2.32 (s, 3H), 2.42 (s br, 1H), 2.58 (s br, 1H), 2.71 (s br, 1H), 2.88 (s br, 2H), 3.17 (s br, 1H), 3.26 (s br, 1H), 3.51 (s, 2H), 3.82 (s br, 1H), 3.89 (s br, 1H), 4.17 (s br, 1H), 4.65 (s br, 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.

9-(1-Benzylpiperidin-4-ylamino)-acridine-4-carboxylic Acid Diethylamide (15b). Yield = 17.7%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.17 (s br, 3H), 1.30 (s br, 3H), 1.75 (m br, 2H), 2.07 (m br, 4H), 2.88 (m br, 2H), 3.41 (s br, 2H), 3.52 (s, 2H), 3.63 (s br, 2H), 3.87 (s br, 1H), 4.72 (s br, 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.

[9-(1-Benzylpiperidin-4-ylamino)-acridin-4-yl]-morpholin-4-yl-methanone (15c). Yield = 60.7%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.74 (m br, 2H), 2.07 (m br, 4H), 2.88 (s br, 2H), 3.17 (s br, 1H), 3.23 (s br, 1H), 3.46 (s br, 1H), 3.51 (s br, 2H), 3.82 (m br, 4H), 4.01 (s br, 1H), 4.26 (s br, 1H), 4.68 (s br, 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.

{**9-[2-(1H-Indol-3-yl)-ethylamino]-acridin-4-yl**}**-(4-methylpiperazin-1-yl)-methanone (15d).** Yield = 71.2%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 2.18 (s br, 1H), 2.32 (s, 3H), 2.43 (s br, 1H), 2.58 (s br, 1H), 2.69 (s br, 1H), 3.25 (s br, 4H), 3.88 (s br, 1H), 4.2 (s br, 3H), 5.28 (s br, 1H), 7.07 (s br, 1H), 7.15 (t, 1H), 7.25 (m, 3H), 7.42 (d, 1H), 7.61 (m, 2H), 7.64 (d, 1H), 7.97 (m br, 3H), 8.14 (s br, 1H); HRMS ($C_{29}H_{30}N_5O$) $[M + H]^+$: found *m*/*z* 464.2414, calcd 464.2445.

9-[2-(1H-Indol-3-yl)-ethylamino]-acridine-4-carboxylic Acid Diethylamide (15e). Yield = 52.6%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.02 (s br, 3H), 1.41 (s br, 3H), 3.22 (s br, 2H), 3.75 (m br, 2H), 4.18 (s br, 2H), 5.22 (s br, 2H), 7.03 (s br, 1H), 7.14 (t, 1H), 7.25 (m, 3H), 7.40 (d, 1H), 7.56 (m, 2H), 7.65 (d, 1H), 7.96 (m br, 3H), 8.23 (s br, 1H); HRMS ($C_{28}H_{29}N_4O$) $[M + H]^+$: found *m*/*z* 437.2320, calcd 437.2336.

{**9-[2-(1H-Indol-3-yl)-ethylamino]-acridin-4-yl**}**-morpholin-4-yl-methanone (15f).** Yield = 72.0%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.23 (s br, 4H), 3.46 (s br, 1H), 3.87 (m br, 4H), 4.19 (s br, 3H), 5.29 (s br, 1H), 7.06 (s br, 1H), 7.16 (t, 1H), 7.25 (m, 3H), 7.41 (d, 1H), 7.62 (m, 2H), 7.65 (d, 1H), 7.95 (m br, 3H), 8.18 (s br, 1H); HRMS ($C_{28}H_{27}N_4O_2$) $[M + H]^+$: found *m*/*z* 451.2106, calcd 451.2128.

[9-(1-Benzylpiperidin-4-ylamino)-acridin-3-yl]-(4-methylpiperazin-1-yl)-methanone (15g). Yield = 40.1%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.76 (m br, 2H), 2.08 (m br, 4H), 2.34 (s, 3H), 2.41 (s br, 2H), 2.54 (s br, 2H), 2.88 (s br, 2H), 3.52 (s, 2H), 3.61 (s br, 2H), 3.88 (s br, 3H), 4.74 (s br, 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.

9-(1-Benzylpiperidin-4-ylamino)-acridine-3-carboxylic Acid Diethylamide (15h). Yield = 10.4%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.17 (s br, 3H), 1.30 (s br, 3H), 1.75 (m br, 2H), 2.07 (m br, 4H), 2.88 (m br, 2H), 3.41 (s br, 2H), 3.52 (s, 2H), 3.63 (s br, 2H), 3.87 (s br, 1H), 4.72 (s br, 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.

{**9-[2-(1H-Indol-3-yl)-ethylamino]-acridin-3-yl**}**-(4-methylpiperazin-1-yl)-methanone (15i).** Yield = 29.6%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 2.33 (s, 3H), 2.39 (s br, 2H), 2.52 (s br, 2H), 3.25 (t, 2H), 3.56 (s br, 2H), 3.87 (s br, 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 (s br, 1H); HRMS ($C_{29}H_{30}N_5O$) $[M + H]^+$: found *m*/*z* 464.2414, calcd 464.2445.

9-[2-(1H-Indol-3-yl)-ethylamino]-acridine-3-carboxylic Acid Diethylamide (15j). Yield = 45.5%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.14 (s br, 3H), 1.30 (s br, 3H), 3.32 (s br, 4H), 3.60 (s br, 2H), 4.29 (s br, 2H), 7.13 (m, 2H), 7.24 (m, 3H), 7.43 (d, 1H), 7.59 (t, 1H), 7.63 (d, 1H), 7.84 (s br, 1H), 7.94 (d, 1H), 8.06 (d, 1H), 8.10

(s, 1H), 8.48 (s br, 1H); HRMS ($C_{28}H_{29}N_4O$) $[M + H]^+$: found *m*/*z* 437.2308, calcd 437.2336.

[9-(1-Benzylpiperidin-4-ylamino)-acridin-2-yl]-(4-methylpiperazin-1-yl)-methanone (15k). Yield = 73.6%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.75 (m br, 2H), 2.08 (m br, 4H), 2.35 (s, 3H), 2.48 (s br, 4H), 2.88 (s br, 2H), 3.52 (s, 2H), 3.61 (s br, 2H), 3.88 (m br, 3H), 4.84 (s br, 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.

9-(1-Benzyl-piperidin-4-ylamino)-acridine-2-carboxylic Acid Diethylamide (15l). Yield = 50.7%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.23 (s br, 6H), 1.75 (m br, 2H), 2.07 (m br, 4H), 2.87 (m br, 2H), 3.41 (s br, 2H), 3.51 (s, 2H), 3.63 (s br, 2H), 3.88 (s br, 1H), 4.76 (s br, 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.

{9-[2-(1H-Indol-3-yl)-ethylamino]-acridin-2-yl}-(4-methylpiperazin-1-yl)-methanone (15m). Yield = 60.3%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 2.32 (s, 3H), 2.42 (s br, 4H), 3.23 (t, 2H), 3.49 (s br, 2H), 3.82 (s br, 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 (s br, 1H); HRMS ($C_{29}H_{30}N_5O$) $[M + H]^+$: found *m*/*z* 464.2415, calcd 464.2445.

9-[2-(1H-Indol-3-yl)-ethylamino]-acridine-2-carboxylic Acid Diethylamide (15n). Yield = 82.8%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.11 (s br, 3H), 1.28 (s br, 3H), 3.22 (t, 2H), 3.26 (s br, 2H), 3.58 (s br, 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 (s br, 2H), 8.43 (s br, 1H); HRMS ($C_{28}H_{29}N_4O$) $[M + H]^+$: found *m*/*z* 437.2310, calcd 437.2336.

2-(4-*tert*-Butylphenyl)-4-chloroquinoline (20). Anthranilic acid (10.9 mmol) and acetophenone (10.9 mmol) were heated to reflux in ~50 mL of $POCl_3$ for 2 h. Excess $POCl_3$ was distilled off under vacuum, and the remaining was oily solid was poured into a mixture of 20 mL of NH_4OH and 100 mL of ice. When the mixture had melted, it was extracted with EtOAc. TLC indicated several products using 15:1 hexane:EtoAc. The same ratio was used for purification via silica chromatography. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.38 (s, 9H), 7.55 (d, 2H), 7.60 (t, 1H), 7.76 (t, 1H), 7.96 (s, 1H), 8.07 (d, 2H), 8.17 (d, 1H), 8.22 (d, 1H).

2-(4-*tert*-Butylphenyl)-4-(4-methylpiperazin-1-yl)-quinoline (21). 2-(4-*tert*-Butyl-phenyl)-4-chloro-quinoline (**20**) was dissolved in *N*-methylpiperazine and refluxed overnight. The reaction was cooled and placed on a vacuum pump to remove excess amine. The resulting solid was purified using silica gel chromatography eluting with 20:1 CH_2Cl_2:MeOH. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.37 (s, 9H), 2.44 (s, 3H), 2.74 (s br, 4H), 3.33 (s br, 4H), 7.29 (s, 1H), 7.44 (t, 1H), 7.53 (d, 2H), 7.65 (t, 1H), 8.01 (d, 3H), 8.11 (d, 1H); HRMS ($C_{24}H_{30}N_3$) $[M + H]^+$: found *m*/*z* 360.2536, calcd 360.2434.

2-Amino-*N*-methoxy-*N*-methylbenzamide (24). *N*,*O*-Dimethylhydroxylamine·HCl (**23**) (6.13 mmol) was dissolved in 10 mL of 90% ethanol (aq). Triethylamine (9.20 mmol) was then added and stirred at room temperature for 10 min. Next, isatoic anhydride (**22**) (9.50 mmol) was added in portions. The reaction was heated to reflux and stirred for ~2 h and then cooled. Ethanol was removed by rotovap, and the reaction was poured into a 50/50 mix of saturated $NaHCO_3$/ice (~40 mL), then extracted with EtOAc, dried over $MgSO_4$, and condensed. Product was purified on a long column using a 20:1 CH_2Cl_2:MeOH elution mixture. Fractions containing product were concentrated, resulting in a light tan oil. Yield = 51.5%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.35 (s, 3H), 3.60 (s, 3H), 4.64 (s br, 2H), 6.70 (d, 2H), 7.18 (t, 1H), 7.36 (t, 1H).

(2-Aminophenyl)-(4-*tert*-butylphenyl)-methanone (26). 2-Amino-*N*-methoxy-*N*-methyl-benzamide (**24**) (5.55 mmol) and 1-bromo-4-*tert*-butylbenzene (**25**) (5.55 mmol) were dissolved in dry THF and placed under argon. The reaction mixture was then cooled to −78 °C and vigorously stirred. Next, 2.87 M *n*-butyllithium in cyclohexane (11.1 mmol) was added dropwise, and the reaction was stirred for ~30 min at −78 °C. Next, 1 M HCl (aq) (~15 mL) was added, and the reaction was allowed to warm to room temperature. The reaction was then extracted with EtOAc, washed with water, washed with brine, dried with $MgSO_4$, and concentrated. Product was purified on a silica column using 20:1 CH_2Cl_2:MeOH as the eluting solvent. Fractions containing product were concentrated, resulting in a yellow solid. Yield = 53.8%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.36 (s, 9H), 6.01 (s br, 2H), 6.61 (t, 1H), 6.73 (d, 1H), 7.28 (t, 1H), 7.46 (d, 2H), 7.49 (d, 1H), 7.60 (d, 2H).

(4-*tert*-Butylphenyl)-(2-isopropenylaminophenyl)-methanone (27). Acetyl chloride (4.74 mmol) was slowly added to a stirring solution of (2-amino-phenyl)-(4-*tert*-butyl-phenyl)-methanone (**26**) (3.95 mmol) in CH_2Cl_2 at 0 °C. The reaction was allowed to warm to room temperature and stirred overnight. The reaction was quenched with saturated $NaCO_3$ (~15 mL) and extracted with CH_2Cl_2, washed with brine, dried over $MgSO_4$, and concentrated. Product was purified on a silica column using 40:1 CH_2Cl_2:MeOH as the eluting solvent. Fractions containing product were concentrated and recrystallized in hexane, resulting in a pale yellow solid. Yield = 50.5%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.37 (s, 9H), 2.17 (s, 3H), 7.08 (t, 1H), 7.50 (d, 2H), 7.56 (t, 1H), 7.58 (d, 1H), 7.66 (d, 2H), 7.60 (d, 1H), 10.74 (s br, 1H).

4-(4-*tert*-Butylphenyl)-1H-quinolin-2-one (28). (4-*tert*-Butyl-phenyl)-(2-isopropenylamino-phenyl)-methanone (**27**) (0.812 mmol) was dissolved in 15 mL of absolute ethanol, and sodium methoxide (2.44 mmol) was added. The reaction was set to reflux for 1 h. The reaction was cooled and concentrated. The product was extracted into CH_2Cl_2 and washed with water, brine, dried over $MgSO_4$, and concentrated. TLC showed relatively clean product eluting with 9:1 CH_2Cl_2:MeOH, so no purification was performed. Yield = 78.8%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.41 (s, 9H), 6.67 (s, 1H), 7.17 (t, 1H), 7.50 (m, 7H), 10.97 (s br, 1H).

4-(4-*tert*-Butylphenyl)-2-chloroquinoline (29). 4-(4-*tert*-Butyl-phenyl)-1H-quinolin-2-one (**28**) (2.41 mmol) was refluxed in 20 mL of $POCl_3$ for 2 h. The reaction started out faint green and turned clear orange after ~1 h. The reaction was cooled and poured over ice. The mixture was then extracted with CH_2Cl_2 (3 × 50 mL), dried over $MgSO_4$, and concentrated. A short column was run to purify product eluting with 9:1 CH_2Cl_2:MeOH. Yield = 79.9%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.41 (s, 9H), 7.35 (s, 1H), 7.49 (m, 5H), 7.74 (t, 1H), 7.95 (d, 1H), 8.08 (d, 1H).

4-(4-*tert*-Butylphenyl)-2-(4-methylpiperazin-1-yl)-quinoline (30). This followed the same procedure as compound **21**. Dihydrochloride salt was made by dissolving the compound in saturated HCl in ethanol and rotovaping. Yield = 73.9%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.41 (s, 9H), 2.37 (s, 3H), 2.57 (s br, 4H), 6.91 (s, 1H), 7.16 (t, 1H), 7.42 (m, 2H), 7.53 (m, 3H), 7.68 (d, 1H), 7.76 (d, 1H); HRMS ($C_{24}H_{30}N_3$) $[M + H]^+$: found *m*/*z* 360.2429, calcd 360.2434.

General Procedure for 1,3,5-Trisubstituted Pyrazolines (31−43). This followed the same procedure as described for pyrazolines of general structure **5**. As part of a focused library, compounds were only characterized by APCI-HRMS for product verification.

1-(4-Nitrobenzoyl)-5-(4-chlorophenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole (31). HRMS ($C_{20}H_{15}ClN_3O_3S$) $[M + H]^+$: found *m*/*z* 412.0620, calcd 412.0528.

1-(Phenylacetyl)-5-(4-nitrophenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole (32). HRMS ($C_{21}H_{18}ClN_2OS$) $[M + H]^+$: found *m*/*z* 381.0676, calcd 381.0833.

1-(4-Chlorophenylacetyl)-5-(4-chlorophenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole (33). HRMS ($C_{21}H_{17}Cl_2N_2OS$) $[M + H]^+$: found *m*/*z* 415.0273, calcd 415.0444.

1-(Trimethylacetyl)-5-(4-chlorophenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole (34). HRMS ($C_{18}H_{20}ClN_2OS$) $[M + H]^+$: found *m*/*z* 347.1005, calcd 347.0990.

1-(4-Chlorophenylacetyl)-5-(4-bromophenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole (35). HRMS ($C_{21}H_{17}BrClN_2OS$) $[M + H]^+$: found *m*/*z* 458.9740, calcd 458.9938.

1-Benzoyl-5-(4-fluorophenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole (36). HRMS ($C_{20}H_{16}FN_2OS$) $[M + H]^+$: found *m*/*z* 351.0815, calcd 351.0972.

1-(4-Chlorophenylacetyl)-5-(4-methoxyphenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole (37). HRMS ($C_{22}H_{20}ClN_2O_2S$) $[M + H]^+$: found *m*/*z* 411.0771, calcd 411.0939.

1-Benzoyl-5-(4-nitrophenyl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazole (38). HRMS ($C_{20}H_{16}N_3O_3S$) $[M + H]^+$: found *m*/*z* 378.1087, calcd 378.0917.

1-Benzoyl-5-(4-chlorophenyl)-3-(5-bromothiophen-2-yl)-4,5-dihydro-1*H*-pyrazole (39). HRMS ($C_{20}H_{15}BrClN_2OS$) $[M + H]^+$: found *m*/*z* 444.9993, calcd 444.9782.

1-Benzoyl-5-(4-chlorophenyl)-3-(phenyl)-4,5-dihydro-1*H*-pyrazole (40). HRMS ($C_{22}H_{18}ClN_2O$) $[M + H]^+$: found *m*/*z* 361.0968, calcd 361.1113.

1-(4-Chlorophenylacetyl)-5-(4-bromophenyl)-3-(furan-2-yl)-4,5-dihydro-1*H*-pyrazole (41). HRMS ($C_{21}H_{17}BrClN_2O_2$) $[M + H]^+$: found *m*/*z* 442.9983, calcd 443.0167.

1-Benzoyl-5-(4-chlorophenyl)-3-(*tert*-butyl)-4,5-dihydro-1*H*-pyrazole (42). HRMS ($C_{20}H_{22}ClN_2O$) $[M + H]^+$: found *m*/*z* 341.1353, calcd 341.1426.

1-(Trimethylacetyl)-5-(4-chlorophenyl)-3-(*tert*-butyl)-4,5-dihydro-1*H*-pyrazole (43). HRMS ($C_{18}H_{26}ClN_2O$) $[M + H]^+$: found *m*/*z* 321.1665, calcd 321.1739.

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Note Added after ASAP Publication. This manuscript was released ASAP on February 24, 2006, with incorrect values for CC_{50} and TI for **5a** and **5b** in Table 1. The correct version was posted on March 1, 2006.

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