Articles

Studies on Pyrrolopyrimidines as Selective Inhibitors of Multidrug-Resistance-Associated Protein in Multidrug Resistance

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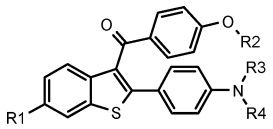
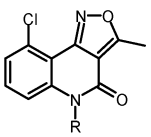
Multidrug resistance mediated by P-glycoprotein (Pgp) or multidrug-resistance-associated protein (MRP) remains a major obstacle for successful treatment of cancer. Inhibition of Pgp and MRP transport is important for high efficacy of anticancer drugs. While several Pgp inhibitors have entered clinical trials, the development of specific MRP1 inhibitors is still in its infancy. In our screening program, we have identified a pyrrolopyrimidine (**4**) as a novel and selective MRP1 inhibitor. Subsequent SAR work on the 4-position of the template revealed the phenethylpiperazine side chain as a potent replacement of the benzylthio group of the lead molecule. Introduction of groups at the 2-position seems to have no detrimental effect on activity. Modifications to the nitrile group at the 7-position resulted in the identification of analogues with groups, such as amides, with superior pharmacokinetic profiles. In vivo efficacy has been demonstrated by xenograft studies on selected compounds.

The appearance of tumor cells resistant to a range of cytotoxic drugs is a serious problem in cancer chemotherapy. This phenomenon is called multidrug resistance (MDR). One form of MDR can be caused by members of the ATP-binding cassette (ABC) family of transport proteins.[1] These are large polytopic membrane proteins that actively transport drugs out of cells, resulting in a decreased intracellular drug concentration. In humans, two ABC transporters have been identified that cause resistance in tumor cells: P-glycoprotein (Pgp) (MDR1)[2] and the multidrug resistance associated protein (MRP1).[3] Pgp transports drugs in an unmodified form, whereas MRP1 transports drugs conjugated to the anionic ligands glutathione (GSH), glucuronide, or sulfate[4] or transports them in an unmodified form, probably together with GSH.[5] Among those cytotoxics transported by MRP1 are various natural product oncolytics, such as vinca alkaloids, epipodophyllotoxins, anthracyclines, and camptothecins,[6] most of which are also substrates for Pgp transport,[7] although taxanes are apparently not subject to MRP1 mediated resistance.[8] Furthermore, MRP1 transports leukotriene C4 (LTC4) as substrate in an ATP-dependent fashion with high efficiency.[9]

The differential expression and tissue/tumor specificities of Pgp and MRP1 have been reviewed recently,[10,11] although it is also known that Pgp and MRP can be overexpressed at the same time in drug-resistant cells.[12] The correlation between drug resistance and expression of the drug efflux pumps, Pgp and MRP1, has spurred considerable efforts in the development of inhibitors of Pgp and MRP1.[13] Recently, Robert has reviewed a number of MDR reversing agents in clinical trials,[14] including MS-209, a dual modulator against both Pgp and MRP.[15] Among the specific potent inhibitors of Pgp entered into clinical evaluation are GF120918,[16] LY335979,[17] OC144-093,[18] and XR9576.[19] However, the development of specific MRP1 modulators is still in its infancy,[13b] although Eli Lilly has reported the raloxifene analogues **1**[20] and isooxazoloquinoline analogues **2**[21] as selective MRP1 inhibitors.



(**1**)



(**2**)

Previously, we have reported the dual inhibitory activities of quinazolinone analogues **3** against Pgp and MRP1.[22] In our continued effort to develop selective MRP1 inhibitors, we screened our in-house chemical library against the target. This led to the identification of the pyrrolopyrimidine analogue **4** as the lead molecule. Compound **4** is a highly selective MRP1 inhibitor with an IC_{50} of 3.9 μM (concentration resulting in 50% inhibition of tritiated daunomycin efflux by cells) in drug accumulation assays using the MRP1 expressing cell line COR.L23/R and with an IC_{50} greater than 50 μM

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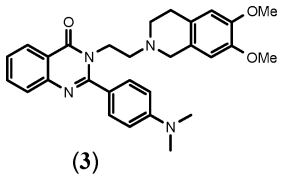
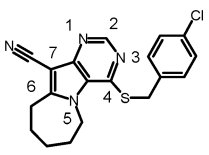


(3)



(4)

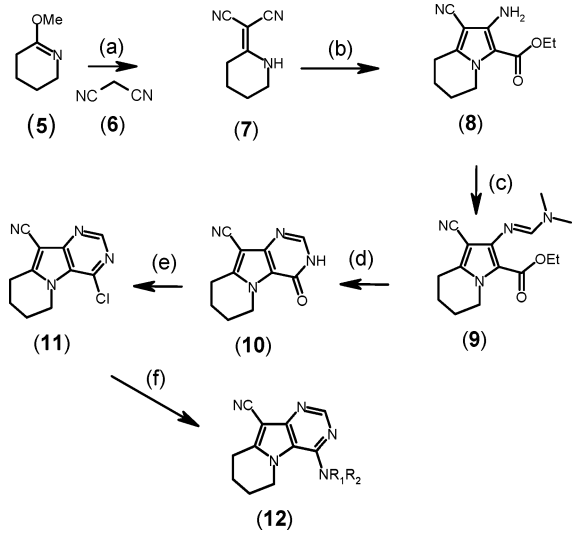
for Pgp activity (EMT6/AR1.0 subline). Here, we report our SAR studies of pyrrolopyrimidine analogues as novel and selective MRP1 inhibitors.

Our lead optimization strategy for this series was initially aimed at the following: (i) replacement of benzylthio group at the 4-position by amines in order to enhance diversity and remove the metabolic liability of the sulfur atom; (ii) modification of the nitrile group at the 7-position into other functional groups to define their importance of binding in this region; (iii) introduction of substituents at the 2-position to identify potential new binding interactions in this area. The above positions were chosen for structural modification because they explore different areas of space. Other considerations were the ease of synthetic access and the availability of reagents and functional groups suitable for manipulation. The common intermediate **11** (Scheme 1) allows rapid exploration of the 4-position, and the 2-position is easily modified as outlined in Scheme 2. A variety of structurally diverse commercially available amines were identified to react with the common intermediate **11** to probe what functionality is tolerated in this region. The nitrile group at the 7-position is a versatile moiety for functional group manipulation as indicated in Scheme 3.

Chemistry

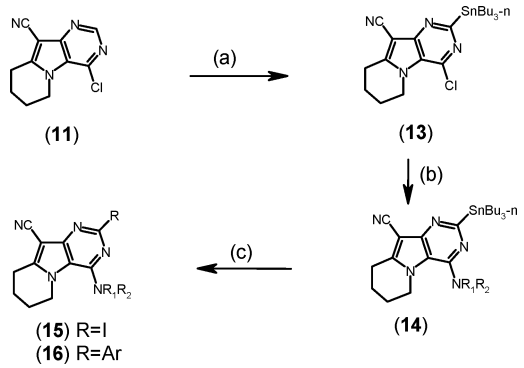
At first, we investigated the possibility of replacing the thiobenzyl side chain with amines because this would allow the rapid introduction of diversity through parallel synthesis. A library of such compounds (**12**) were prepared via the key intermediate, chloropyrimidine **11**, following a literature protocol[23] (Scheme 1).

Scheme 1[a]



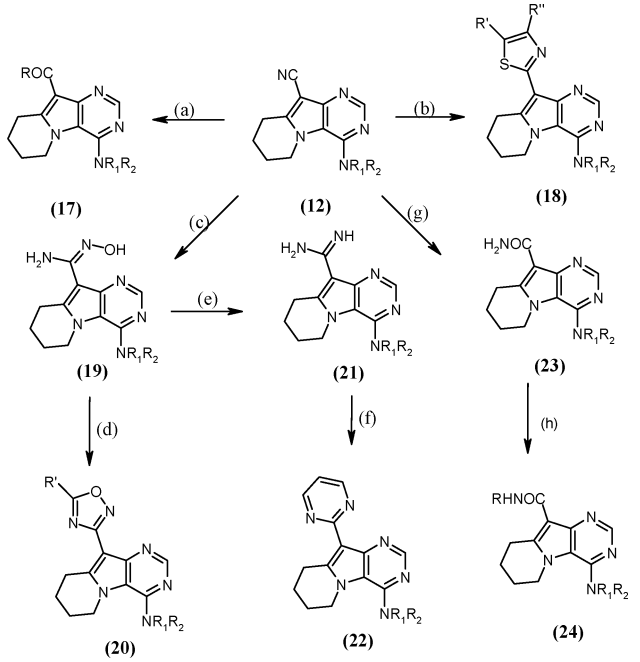
[a] Reagents: (a) EtOH, room temp, 97%; (b) $BrCH_2CO_2Et$, K_2CO_3, DMF, 100 °C, 55%; (c) $(MeO)_2CHN(CH_3)_2$, DMF, 100 °C, 90%; (d) NH_3, EtOH, bomb, 110°C, 80%; (e) $POCl_3$, TEA·HCl (0.4 equiv), reflux, 85%; (f) R_1R_2NH, DMF, 100 °C, 82−95%.

Scheme 2[a]



[a] Reagents: (a) (i) TMP, *n*-BuLi, −78 °C, THF, (ii) *n*-Bu_3SnCl, 70% (two steps); (b) R_1R_2NH, *i*-PrOH, Et_3N, reflux, 72%−95%; (c) I_2, THF, room temp (**15**), 81%; ArI, $Pd(Ph_3P)_4$, CuI, TEA, THF, argon, reflux, 73−85%.

Scheme 3[a]



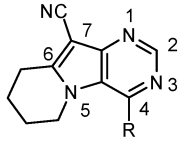
[a] Reagents: (a) RMgBr, THF, 60 °C, 75−85%; (b) (i) NaHS, DMF, 60 °C, 5 days; (ii) R′CH(X)COR″, EtOH, 100 °C, 82−93%; (c) NH_2OH·HCl, K_2CO_3, EtOH, reflux, 82%; (d) RCOCl, 60 °C, 18%; (e) H_2, Pd/C, room temp, 72%; (f) $(MeO)_2CHCH_2CH(MeO)_2$, Dowtherm, 175 °C, 83%; (g) NaOH (1.0 equiv, 1 M), H_2O_2 (27%, 5 equiv), MeOH, room temp, 85%; (h) RCHO, Et_3SiH, TFA, CH_3CN, reflux, 25−90%.

The synthesis of 2-substituted pyrrolopyrimidine analogue is depicted in Scheme 2.

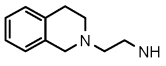
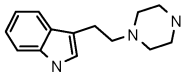
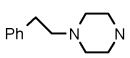
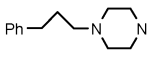
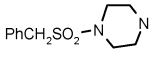
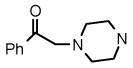
Chloropyrimidine **11** was lithiated with *n*-BuLi in the presence of 2,2,6,6-tetramethylpiperidine (TMP) in THF at −78 °C, followed by quenching with tri-*n*-butyltin chloride to give compound **13** in 70% yield. The subsequent replacement of the chloro group with amines gave the intermediate products **14**. These were converted into either the 2-iodo analogue **15** or 2-aryl analogues **16** under Stille coupling conditions.[24]

To ensure a convergent route to rapid analogue generation, we focused on the transformation of the nitrile group of **12**, as shown in Scheme 3. Thus, a number of functional groups were introduced, including ketones (**17**), thiazoles (**18**), oxadiazoles (**20**), pyrimidines (**22**), and amides (**23**, **24**). The preparation of the

Table 1. Inhibitory Activity[a] of 4-Substituted Compounds in Accumulation Assays for MRP1(L23/R) and Pgp (EMT6/AR1.0)



(12)

Compd	R	IC_{50}(μM) / MRP1[a]	IC_{50}(μM) / Pgp[a]
25	$PhCH_2CH_2NH$	6.3	16.0
26	$PhCH_2CH_2NCH_3$	9.0	33.0
27	$PhCH_2SO_2NH$	20.9	5.4
28		6.35	>50
29		0.6	5.82
30		0.69	9.63
31		7.35	13.0
32		6.65	14.0
33		1.85	23

[a] All the IC_{50} values in this text represent the mean of a minimum of three experiments. Variations among the results were less than 10%.

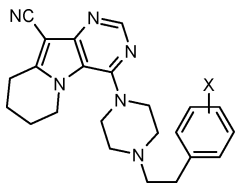
secondary amides (**24**) were achieved from the primary amides (**23**) using modified literature procedures.[25]

Results and Discussion

By use of the synthetic method described in Scheme 1, a library of compounds (**12**) were prepared and evaluated in drug accumulation assays using both MRP1 expressing cell line COR.L23/R and a Pgp expressing cell line EMT6/AR1.0. The initial screening of this library of compounds uncovered a number of active compounds that showed significant MRP1 inhibitory activity and selectivity against Pgp. Some representative examples are illustrated in Table 1.

Replacement of the benzylthio group within lead molecule **4** with primary and secondary amines appeared to retain the MRP1 inhibitory activity and some selectivity against Pgp as represented by compounds **25**, **26**, and **28**. In general, amide or sulfonamide linkages resulted in a decrease of MRP1 activity and poor selectivity over Pgp (**27**). However, compounds bearing a piperazine linkage exhibited significantly improved

Table 2. Inhibitory Activity of Substituted Phenethylpiperazine Compounds in Accumulation Assays for Pgp(EMT6/AR1.0) and MRP1(L23/R)



compd	X	IC_{50} (μM)/MRP1[a]	IC_{50} (μM)/Pgp[a]
30	H	0.69	9.63
34	2-Cl	0.955	10.6
35	3-Cl	0.92	17.9
36	4-Cl	0.399	9.2
37	2-NO_2	1.415	>50
38	3-NO_2	0.41	9.665
39	4-NO_2	0.523	12.94
40	3-MeO	2.30	16.2
41	4-MeO	>50	nd[b]
42	3,4-F,F	0.229	10.52

[a] All the IC_{50} values in this text represent the mean of a minimum of three experiments. Variations among the results were less than 10%. [b] nd: not determined.

MRP1 activity and good selectivity (**29**, **30**). Further modifications of the piperazine ring, such as different size ring structures with or without additional functional groups (data not shown) and its *N*-alkyl side chain, did not give any improvement in potency and selectivity (**31**−**33**). Given that both indole and phenyl analogues **29** and **30** showed similar activity and selectivity, it was decided to examine the SAR of the phenyl ring of analogue **30** in more detail. Some of the data in this area is illustrated in Table 2.

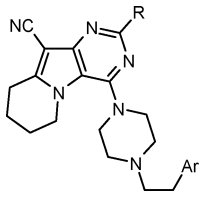
As shown in Table 2, ortho-substituted analogues **34** and **37** appeared to be marginally less active than the parent analogue **30** in MRP1 modulation. Compounds with electron-withdrawing groups at the 3- or 4-position (**36**, **38**, **39**) gave better MRP1 activity than those with electron-donating groups (**40**, **41**). In particular, the 3,4-difluorophenethyl analogue **42** appeared to be 3-fold more potent than the parent compound **30**. Consequently, this side chain and the readily available phenethylpiperazine side chain present in compound **30** were retained for further optimization work in other areas of the lead molecule **4**.

Next, we turned our attention to the SAR studies on 2-substituted pyrrolopyrimidine analogues, which were conveniently prepared according to the chemistry described in Scheme 2. Table 3 exemplifies some of the active analogues in this area.

Compared with the activity of compound **30**, the introduction of iodo and aryl groups at the 2-position of the pyrimidine ring was not detrimental to activity, and the MRP1 selectivity over Pgp was retained (**43**−**45**). The corresponding pyridine analogues (**46**, **47**) showed good MRP1 activity comparable to that of compound **42**, with **47** showing >2-fold improvement in potency. The more sterically hindered 2-methylphenyl-substituted compound **48** showed 3-fold less potency than compound **42**. Therefore, substitution at this position was generally well tolerated and this position was identified as a good point for attaching solubilizing groups without reducing the inherent MRP1activity (results to be published).

Although we had made significant progress in finding selective and potent MRP1 inhibitors, our early evalu-

Table 3. Inhibitory Activity for 2-Substituted Pyrrolopyrimidine Compounds in Accumulation Assays for Pgp(EMT6/AR1.0) and MRP1(L23/R)



compd	R	Ar	IC_{50} (μM)/ MRP1[a]	IC_{50} (μM)/ Pgp[a]
30	H	Ph	0.69	9.63
43	I	Ph	0.887	13.05
44	Ph	Ph	0.715	5.0
45	4-CH_3CONHPh	Ph	0.57	23.7
46	3-Pyr	3,4-(F,F)-Ph	0.218	nd[b]
47	4-Pyr	3,4-(F,F)-Ph	0.105	1.31
48	2-CH_3-Ph	3,4-(F,F)-Ph	0.62	17.99

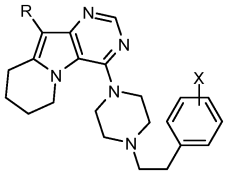
[a] All the IC_{50} values in this text represent the mean of a minimum of three experiments. Variations among the results were less than 10%. [b] nd: not determined.

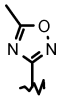
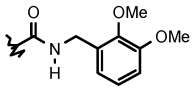
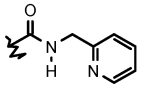
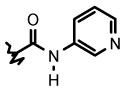
ation of some of the active compounds suggested only a limited degree of metabolic stability with poor pharmacokinetic profiles. Pharmacokinetic studies of two of our early lead compounds, **30** and **42**, showed very poor half-lives of less than 30 min after intravenous administration. We speculated that the nitrile group at the 7-position on template **4** could be a metabolic weak point and sought to identify a replacement for the nitrile group according to the chemistry described in Scheme 3. Some of the examples are shown in Table 4.

Conversion of the nitrile into ketones and the corresponding methyl ester gave compounds **49**−**51** with reduced MRP1 activity when compared with analogue **30**. Transformation of the nitrile into various heterocycles, such as thiazole, oxadiazole, and pyrimidine (**52**−**54**), gave compounds with similar activity. With the more active side chain, 3,4-difluorophenethylpiperazine, at the 4-position, the simple amide analogues **55** and **56** appeared to be 2-fold more potent than the corresponding nitrile analogue **42**. Further parallel synthesis revealed more secondary amides with significantly enhanced potency against MRP1 and good selectivity over Pgp (**57**−**59**). To further demonstrate the MRP1 inhibitory activity, many compounds were subjected to potentiation assays.[26] Table 5 illustrates the data on several of these compounds (**55**−**59**). Most of the compounds showed approximately 100 nM potency (**55**−**58**), while **59** was 2-fold less potent in this particular assay. To summarize the above results, it is clear that the functional groups at the 7-position are critical for activity, probably as hydrogen bond acceptors in pharmacophoric interactions.

It is widely recognized that the cytochrome P450s (CYP) are a major class of oxidative enzymes involved in drug metablolism.[27] Because any potential MRP1 modulators will be coadministered with cytotoxics, it is important to gain some understanding on potential drug−drug interaction of a molecule before it reaches the later stages of drug development. Therefore, we subjected a number of active compounds to CYP assays, particularly CYP 3A4 (Table 5) because it is considered the major oxidative enzyme for cytotoxic drugs and is expressed at a high level relative to the other hepatic

Table 4. Inhibitory Activity for 7-Substituted Pyrrolopyrimidine Compounds in Accumulation Assays for Pgp(EMT6/AR1.0) and MRP1(L23/R)



Compd.	R	X	MRP1 IC_{50} (μM)[a]	Pgp IC_{50} (μM)[a]
30	CN	H	0.69	9.63
49		H	2.8	>50
50		H	1.37	10.7
51		H	0.91	16.5
52		H	0.533	6.0
53		H	0.65	3.7
54		H	0.675	>50
55	$CONH_2$	3,4-F,F	0.138	>50
56	CONHEt	3,4-F,F	0.12	4.79
57		3,4-F,F	0.043	3.7
58		3,4-F,F	0.061	3.4
59		3,4-F,F	0.076	4.95

[a] All the IC_{50} values in this text represent the mean of a minimum of three experiments. Variations among the results were less than 10%.

Table 5. Inhibitory Activity of Compounds (**55**−**59**) in MRP Potentiation Assay (EC_{50}) and Drug Metabolism Studies with CYP 3A4 Assay (IC_{50})

compd	EC_{50} (μM, PA)[a]	IC_{50} (μM, CYP3A4)[a]
55	0.117	45.8
56	0.10	>100
57	0.15	2.44
58	0.097	30.15
59	0.235	9.73

[a] All the EC_{50} and IC_{50} values in this text represent the mean of a minimum of three experiments. Variations among the results were less than 10%.

CYPs.[28] As the data in Table 5 show, most of these compounds showed little inhibition of CYP 3A4 [29] and these compounds were further evaluated in pharmaco-

Table 6. Pharmacokinetic Studies in Balb/C Mice[a]

dose (mg/kg)	route	C_{max} (μM)	AUC (μg·h/mL)	$t_{1/2}$ (h)	F (%)
		Compound **55**			
20	iv	28	25	1.47	
20	ip	4.43	13.06	6.35	52
50	po	8.37	44.92	6.86	71.6
		Compound **56**			
20	iv	18.31	16.94	2.3	
20	ip	7.34	14.55	1.84	85
50	po	5.73	21.12	7.9	50
		Compound **57**			
20	iv	4	12	0.8	
		Compound **58**			
20	iv	11.39	7.38	2.5	
		Compound **59**			
20	iv	24.49	40.65	6.72	
50	ip	32.49	46.31	4.48	45.6
50	po	14.26	56.37	3.75	55.5

[a] C_{max}, maximum plasma concentration; AUC, area under the concentration−time curve; F, % bioavailability.

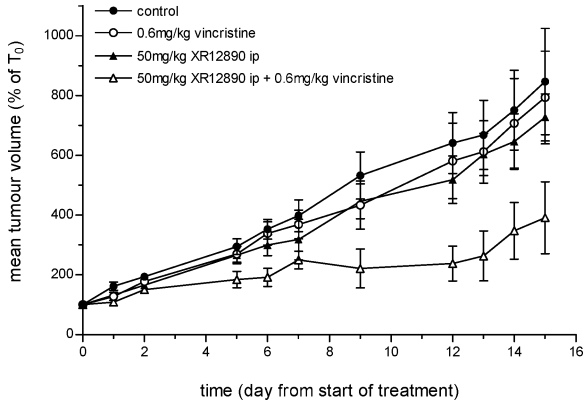


Figure 1. Xenograft curves depicting tumor growth inhibition of subcutaneous COR L23/R cell in CD1 NUDE mice under coadminstration of compound **55**, XR12890, with vincristine.

kinetics studies. This was carried out with Balb/C mice dosed at either 20 or 50 mg/kg intravenously (iv), intraperitoneally (ip), or orally (po) (Table 6).

Compounds **55**, **56**, and **59** showed oral bioavailability of >50% when dosed at 50 mg/kg (Table 6). Moreover, the plasma concentrations of these compounds were 10-fold greater than their corresponding IC_{50} values for several hours after dosing. When given iv at 20 mg/kg, compound **57** had the lowest half-life and C_{max} of the compounds in this series and had a less attractive profile than other members of this series.

To assess the in vivo efficacy of this class of compounds, several compounds were chosen on the basis of their pharmacokinetic data for in vivo xenograft studies with CD1 athymic mice bearing subcutaneous COR L23/R tumors. Figure 1 illustrates the xenograft curves for tumor growth inhibition of one of the compounds (**55**), XR12890. The antitumor activity of vincristine (0.6 mg/kg iv, q5d × 2) was significantly ($p < 0.01$) potentiated by the coadminstration of 50 mg/kg of XR12890 intraperitoneally or 100 mg/kg orally (graph not shown). The maximum T/C percent ratios for tumor volumes in animals treated with vincristine plus XR12890 ip or po were 37% or 35%, respectively. In contrast, vincristine or XR12890 alone had no significant effect on tumor growth in this MRP1-resistant tumor model. The treatments with the combination schedules were well tolerated (loss of body weight less than 15%).

Conclusion

In the present report we have described some synthesis procedures and SAR studies of our lead MRP1 inhibitor **4** through structural modifications at the 2-, 4-, and 7-position of the pyrrolopyrimidine template. Through this study, we established that substitution at the 2-position was not detrimental to activity and 3,4-difluorophenylethylpiperazine was a well tolerated side chain at the 4-position, whereas various H-bond acceptor groups were identified at the 7-position to confer activity through crucial pharmacophoric interactions. As a result, a number of novel, potent, and selective MRP1 modulators with good pharmacokinetic profiles were identified. Their in vivo efficacies were also demonstrated in xenograft models, exemplified by compound **55**, XR12890. Further in vivo studies with different cytotoxic agents and this compound will be published elsewhere.

Experimental Section

Methods and Materials. Reagents, starting materials, and solvents were purchased from common commercial suppliers and used as received or distilled from the appropriate drying agent. Reactions requiring anhydrous conditions were performed under an atmosphere of nitrogen or argon. Precoated aluminum-backed silica gel 60 F_{254} plates with a layer thickness of 0.25 mm were used for thin-layer chromatography, and the stationary phase for preparative column chromatography using medium pressure was silica gel 60, mesh size 40−60 μm from E. Merck, Darmstadt, Germany.

NMR spectra were obtained using a Bruker ACF 400 operating at 400 MHz, and the ^{1}H shift values, in ppm, were calibrated to that of residual $CHCl_3$ in $CDCl_3$ at 7.26 ppm. Mass spectra were obtained in the indicated mode using a Finnigan SSQ 710L machine. Melting points were determined using an electrothermal 9100 series apparatus.

The compound purity on all compounds tested in biological systems was assessed as being >95% using HPLC, using both a water/acetonitrile gradient containing 0.02% TFA at 30 °C on a Waters symmetry C_{18}, 5 μM, 150 mm × 3.9 mm column and a water/actonitrile gradient containing 0.05% phosphoric acid at 30 °C on a LiChrospher RP-8, 5 μM, 250 mm × 4.6 mm column. UV photodiode array detection was applied. Microanalyses were performed on a representation of the compounds by MEDAC Ltd., U.K. Where analyses are indicated only by the symbols of the elements, results obtained were within 0.4% of the theoretical values.

Chemistry. General Procedure for the Synthesis of Pyrrolopyrimidines 12 and Compounds 25, 26, 28−42, Exemplified by Preparation of Compound 30. 4-(4-Phenethylpiperazine-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (30). A mixture of 4-chloro-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (**11**) (80 mg, 0.343 mmol), 1-(2-phenylethyl)piperazine (1.1 equiv, 72 mg), and triethylamine (1.1 equiv, 53 μL, 0.377 mmol) was heated in DMF (0.5 mL) at 100 °C for 2 h. The reaction mixture was cooled and poured onto ice/water and the precipitated solid was collected by filtration to yield the desired title compound (94 mg, 70%). Mp 176−177 °C; ^{1}H NMR ($CDCl_3$) δ 1.97−2.13 (m, 4H, 2 × CH_2), 2.64−2.80 (m, 6H, 3 × CH_2), 2.81−2.92 (m, 2H, CH_2), 3.22 (2H, t, J = 6.4 Hz), 3.38−3.50 (m, 4H, 2 × CH_2), 4.45 (2H, t, J = 5.5 Hz), 7.16−7.36(m, 5H, 5 × ArH); MS m/z 387 $(M + H)^+$.

4-Phenethylamino-4a,5,6,7,8,9a-hexahydro-1,3,4b-triazafluorene-9-carbonitrile (25). Mp 182−183 °C; ^{1}H NMR ($CDCl_3$) δ 1.84−1.94 (m, 2H, CH_2), 2.0−2.10 (m, 2H, CH_2), 3.02 (t, 2H, J = 6.6 Hz, CH_2), 3.08 (t, 2H, J = 6.3 Hz, CH_2), 3.99 (q,

2H, J = 5.2 Hz), 4.02 (t, 2H, J = 6.2 Hz), 4.84 (brs, 1H, NH), 7.18−7.40 (5H, m, 5 × ArH), 8.45 (s, 1H, ArH); MS m/z 318 $(M + H)^+$.

4-(Methylphenethylamino)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (26). Mp 128−129 °C; ^{1}H NMR ($CDCl_3$) δ 1.90 (m, 4H, 2 × CH_2), 2.95 (t, 2H, CH_2), 2.98 (s, 3H, CH_3), 3.15 (t, 2H, CH_2), 3.65 (t, 2H, CH_2), 4.00 (t, 2H, CH_2), 7.10−7.25 (m, 5H, ArH), 8.60 (s, 1H, PyrmH); MS m/z 332 $(M + H)^+$.

***N*-(9-Cyano-5,6,7,8-tetrahydro-1,3,4b-triazafluoren-4-yl)-*C*-phenylmethanesulfonamide (27).** To a solution of 4-chloro-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (**11**) (0.073 g, 0.343 mmol) in dioxane (6 mL) was added ethanol (12 mL) saturated with ammonia at 0 °C. The mixture was heated with stirring in a sealed tube at 140 °C for 20 h. The reaction mixture was evaporated in vacuo, the residue was triturated with hot methanol, and the creamy solid was filtered as 4-amino-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (415 mg, 65%). A mixture of the above product (73 mg, 0.343 mmol) and benzylsulfonyl chloride (1.5 equiv, 98 mg) and DMAP (cat.) was heated in pyridine (1.5 mL) at 50−70 °C for 4 h. Pyridine was removed in vacuo, and the residue was taken up in ethyl acetate, washed with saturated $NaHCO_3$, dried over $MgSO_4$, and concentrated in vacuo. After purification on column chromatography with dichloromethane, brownish crystals were obtained (20 mg, 16%). Mp 128−129 °C; ^{1}H NMR ($CDCl_3$) δ 1.95−2.00 (m, 2H, CH_2), 2.03−2.10 (m, 2H, CH_2), 3.10 (t, 2H, J = 6.4 Hz, CH_2), 4.40 (s, 2H, CH_2), 4.45 (t, 2H, J = 6.1 Hz, CH_2), 7.20−7.30 (m, 3H, 3 × ArH), 7.45 (d, 2H, J = 8.0 Hz), 7.60 (s, 1H, ArH), 11.55 (br s, 1H, NH); MS m/z 368 $(M + H)^+$.

4-[2-(3,4-Dihydro-1*H*-isoquinolin-2-yl)ethylamino]-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (28). ^{1}H NMR ($CDCl_3$) δ 1.70−1.80 (m, 4H, 2 × CH_2), 2.85−2.93 (m, 6H, 3 × CH_2), 3.02 (t, 2H, CH_2), 3.70−3.75 (m, 4H, 2 × CH_2), 4.20 (t, 2H, CH_2), 6.25 (br s, 1H, NH), 7.02 (d, 1H, J = 7.5 Hz, ArH), 7.12−7.20 (m, 3H, ArH), 8.45 (s, 1H, ArH); MS m/z 373 $(M + H)^+$.

4-{4-[2-(1*H*-Indol-3-yl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (29). Mp 223−232 °C; ^{1}H NMR (DMSO-d_6) δ 1.85−2.05 (m, 4H, CH_2), 2.6−2.75 (m, 6H, 3 × CH_2), 2.87 (t, 2H, J = 8.4 Hz), 3.15 (t, 2H, J = 7.3 Hz), 3.32−3.4 (m, 4H, 2 × CH_2), 4.32−4.40 (m, 2H, CH_2), 7.00 (t, 1H, J = 7.1 Hz), 7.05 (t, 1H, J = 7.1 Hz, ArH), 7.19 (s, 1H, ArH), 7.32 (d, 1H, J = 8.1 Hz, ArH), 7.53 (d, 1H, J = 7.8 Hz), 8.48 (s, 1H, ArH), 10.78 (s, 1H, ArH); MS m/z 426.2 $(M + H)^+$.

4-[4-(3-Phenylpropyl)piperazin-1-yl]-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (31). Mp 156−158 °C; ^{1}H NMR ($CDCl_3$) δ 1.8−1.92 (m, 2H), 2.99−2.15 (m, 4H, CH_2), 2.39−2.50 (t, 2H, J = 7.6 Hz, CH_3), 2.59−2.72 (m, 6H, 3 × CH_2), 3.18−3.25 (t, 2H, J = 6.3 Hz), 3.38−3.45 (m, 4H, 2 × CH_2), 4.31−4.42 (t, 2H, J = 6.0 Hz), 7.13−7.32 (m, 5H, 5 × ArH), 8.62 (s, 1H, ArH); MS m/z 401.3 $(M + H)^+$.

4-(4-Phenylmethanesulfonylpiperazin-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (32). Mp 239−241 °C; ^{1}H NMR (DMSO-d_6) δ 1.85−1.97 (m, 4H, CH2), 3.10−3.18 (t, 2H, J = 6.2 Hz), 3.25−3.35 (m, 8H, 4 × CH2), 3.21−3.3(m, 2H, CH_2), 4.3−4.39 (2H, t, J = 5.8 Hz), 4.50 (s, 2H, CH_2), 7.32−7.45 (m, 5H, 5 × ArH), 8.51 (s, 1H, ArH); MS m/z 437.3 $(M + H)^+$. Anal. ($C_{22}H_{24}N_6O_2S\cdot0.5H_2O$) C, H, N.

4-[4-(2-Oxo-2-phenylethyl)piperazin-1-yl]-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (33). Mp 171−173 °C; ^{1}H NMR (DMSO-d_6) δ 1.85−1.97 (m, 4H, 2 × CH_2), 2.70−2.76 (m, 4H, 2 × CH_2), 3.09−3.15 (2H, t, J = 6.2 Hz, CH_2), 3.41−3.49 (m, 4H, 2 × CH_2), 3.95 (s, 2H, CH_2), 4.32−4.4 (t, 2H, J = 5.7 Hz, CH_2), 7.52 (2H, t, J = 7.5 Hz, ArH), 7.62 (1H, t, J = 7.4 Hz, ArH), 8.02 (d, 2H, J = 7.2 Hz, ArH), 8.5 (s, 1H, ArH); MS m/z 401.3 $(M + H)^+$.

4-{4-[2-(2-Chlorophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (34). Mp 156−158 °C; ^{1}H NMR ($CDCl_3$) δ 1.99−2.12 (m, 4H, 2 × CH_2), 2.67−2.78 (m, 6H, 2 × CH_2), 2.98−3.07 (m, 2H, CH2), 3.20 (t, 2H, J = 6.4 Hz, CH_2), 3.42 (t, 4H, J = 4.8 Hz, CH_2), 4.35 (t, 2H, J = 5.9 Hz, CH_2), 7.12−7.25 (m, 2H 2 × ArH), 7.21−7.34 (1H (under solvent peak), ArH), 7.33−7.40 (d, 1H, J = 7.6 Hz, ArH), 8.61 (s, 1H, ArH); MS m/z 421.3 $(M + H)^+$.

{4-[2-(3-Chlorophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (35). Mp 205−206 °C; ^{1}H NMR ($CDCl_3$) δ 1.88−2.18 (m, 4H, 2 × CH_2), 2.48−2.98 (m, 8H, 4 × CH_2), 3.10−3.52 (m, 6H, 3 × CH_2), 4.22−4.42 (m, 2H, CH_2), 7.00−7.35 (m, 4H, 4 × ArH), 8.60 (s, 1H, ArH); MS m/z 421.3 $(M + H)^+$.

4-{4-[2-(4-Chlorophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (36). ^{1}H NMR ($CDCl_3$) δ 1.99−2.12 (m, 4H, 2 × CH_2), 2.62−2.75 (m, 6H, 3 × CH_2), 2.77−2.86 (m, 2H, CH_2), 3.22 (t, 2H, J = 6.3 Hz, CH_2), 3.42 (t, 4H, J = 4.8 Hz, 2 × CH_2), 4.35 (2H, t, J = 5.9 Hz, CH_2), 7.12−7.20 (d, 2H, J = 8.4 Hz, CH_2), 7.22−7.31 (d, 2H, 2 × ArH), 8.61 (s, 1H, ArH); MS m/z 421.2 $(M + H)^+$.

4-{4-[2-(2-Nitrophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (37). ^{1}H NMR ($CDCl_3$) δ 1.99−2.12 (m, 4H, CH_2), 2.67−2.78 (m, 6H, 3 × CH_2), 3.14 (t, 2H, J = 6.6 Hz, CH_2), 3.20 (t, 2H, J = 6.6 Hz, CH_2), 3.42 (t, 4H, J = 4.8 Hz, 2 × CH_2), 4.35 (2H, t, J = 5.9 Hz, CH_2), 7.35−7.44 (m, 2H, 2 × ArH), 7.52 (t, 1H, J = 8.7 Hz, ArH), 7.9−7.98 (d, 1H, J = 8.2 Hz), 8.61 (s, 1H, ArH); MS m/z 432.2 $(M + H)^+$.

4-{4-[2-(3-Nitrophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (38). Mp 194−195 °C; ^{1}H NMR ($CDCl_3$) δ 1.99−2.15 (m, 4H, CH_2), 2.69−2.83 (m, 6H, 3 × CH_2), 2.96 (t, 2H, J = 7.6 Hz, CH_2), 3.20 (t, 2H, J = 6.4 Hz, CH_2), 3.38−3.58 (m, 4H, 2 × CH_2), 4.36 (t, 2H, J = 5.6 Hz, CH_2), 7.47 (t, 1H, J = 7.9 Hz, ArH), 7.59 (1H, d, J = 7.6 Hz, ArH), 8.10 (t, 2H, J = 8.0 Hz, 2 × ArH), 8.62 (s, 1H, ArH); MS m/z 432.3 $(M + H)^+$.

4-{4-[2-(4-Nitrophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (39). ^{1}H NMR (DMSO-d_6) δ 1.87−2.0 (m, 4H, CH_2), 2.62−2.7 (m, 4H, 2 × CH_2), 2.90 (t, 2H, J = 7.2 Hz, CH_2), 3.12 (2H, t, J = 6.5 Hz, CH_2), 3.25−3.34 (m, 6H, 3 × CH_2), 4.34 (t, 2H, J = 5.8 Hz, CH_2), 7.54 (d, 2H, J = 8.7 Hz, 2 × ArH), 8.13 (2H, t, J = 8.7 Hz, 2 × ArH), 8.50 (s, 1H, ArH); MS m/z 432.3 $(M + H)^+$.

4-{4-[2-(3-Methoxyphenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (40). Mp 181−182 °C; ^{1}H NMR ($CDCl_3$) δ 1.97−2.11(m, 4H, CH_2), 2.62−2.76 (m, 6H, 3 × CH_2), 2.77−2.87 (m, 2H, CH_2), 3.21 (t, 2H, J = 6.3 Hz, CH_2), 3.36−3.50 (m, 4H, CH_2), 3.80 (s, 3H, CH_3), 4.36 (2H, t, J = 5.4 Hz, CH_2), 6.70−6.86 (m, 3H, 3 × ArH), 7.21 (1H, t, J = 7.7 Hz, ArH), 8.63 (s, 1H, ArH); MS m/z 417.3 $(M + H)^+$.

4-{4-[2-(4-Methoxyphenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (41). ^{1}H NMR (DMSO-d_6) δ 1.88−2.01 (m, 4H, 2 × CH_2), 2.52−2.59 (m, 2H, CH_2), 2.59−2.68 (m, 2H, CH_2), 2.68−2.74 (2H, m, CH_2), 3.12 (t, 2H, J = 6.2 Hz, CH_2), 3.22−3.41 (m, 6H, 3 × CH_2), 3.69 (s, 3H, CH_3), 4.35 (t, 2H, J = 5.0 Hz), 6.82 (d, 2H, J = 8.7 Hz, ArH), 7.16 (t, 2H, J = 8.7 Hz, ArH), 8.45 (s, 1H, ArH); MS m/z 417.3 $(M + H)^+$.

4-{4-[2-(3,4-Difluorophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (42). ^{1}H NMR ($CDCl_3$) δ 1.92−2.12 (4H, m, 2 × CH_2), 2.55−2.7 (m, 6H, 3 × CH_2), 2.72−2.82 (m, 2H, CH_2), 3.21 (t, 2H, J = 6.4 Hz), 3.42 (t, 4H, J = 4.8 Hz, 2 × CH_2), 4.35 (2H, t, J = 5.9 Hz), 6.83−6.93 (m, 1H, ArH), 6.98−7.1 (2H, m, 2 × ArH), 8.61 (s, 1H, ArH); MS m/z 423.3 $(M + H)^+$.

4-Chloro-2-tributylstannanyl-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (13). *n*-BuLi (28 mL, 69.83 mmol, 2.5 M solution in hexane) was added to a solution of 2,2,6,6-tetramethylpiperidine (11.78 mL, 69.83 mmol) in THF (100 mL) at −78 °C. To this was added 4-chloro-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (3.24 g, 13.97 mmol) in THF (100 mL). After the mixture was stirred for 30 min, tributyltin chloride (18.95 mL, 69.83 mmol) was added. After 1 h, the reaction mixture was quenched by addition of ammonium chloride solution. The organics were extracted into

ethyl acetate and dried ($MgSO_4$) and the solvent was removed in vacuo to yield an orange solid (5.1 g, 70%, used crude in next step).

General Procedure for the Synthesis of 14. 4-{4-Phenethylpiperazin-1-yl}-2-tributylstannanyl-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (14a). 4-Chloro-2-tributylstannanyl-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (**13**) and 1-[2-phenethyl]piperazine were reacted together with the standard protocol described above to yield 4-{4-phenethylpiperazin-1-yl}-2-tributylstannanyl-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (70% yield). ^{1}H NMR ($CDCl_3$) δ 0.90−1.70 (m, 29H, 3 × *n*-Bu), 2.00 (m, 4H, 2 × CH_2), 2.70 (m, 6H, 3 × CH_2), 2.87 (m, 2H, CH_2), 3.18 (t, 2H, J = 6.6 Hz, CH_2), 3.42 (m, 4H, 2 × CH_2), 4.35 (t, 2H, J = 5.3 Hz, CH_2), 7.30 (m, 5H, ArH); MS *m*/*z* 676.5 $(M + H)^+$.

2-Iodo-4-(4-phenethylpiperazin-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (43). A mixture of 4-{4-phenethylpiperazin-1-yl}-2-tributylstannanyl-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (**14a**) (200 mg, 0.384 mmol) and I_2 (1.5 equiv) in THF (2 mL) was stirred at room temperature for 2 h. The reaction was then quenched with saturated $Na_2S_2O_3$, the mixture was extracted with dichloromethane, washed with sodium bicarbonate solution, and dried ($MgSO_4$), and the solvent was removed in vacuo to yield the crude product, which was purified using flash chromatography (EtOAc/DCM, 1:1) to yield the title compound (**43**) (0.106 g, 70%). ^{1}H NMR ($CDCl_3$) δ 1.90−2.00 (m, 4H, 2 × CH_2), 2.60−2.65 (m, 6H, 3 × CH_2), 2.75 (m, 2H, CH_2), 3.10 (t, 2H, CH_2), 3.35−3.40 (m, 4H, 2 × CH_2), 4.20 (t, 2H, CH_2), 7.10−7.30 (m, 5H, 5 × ArH); MS *m*/*z* 513.2 $(M + H)^+$.

General Procedure for the Synthesis of Compounds 44−48 Is Exemplified by the Method for 46. 4-{4-[2-(3,4-Difluorophenyl)ethyl]piperazin-1-yl}-2-pyridin-3-yl-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (46). A mixture of 4-{4-[2-(3,4-difluorophenyl)ethyl]piperazin-1-yl}-2-tributylstannanyl-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (**14b**) (360 mg, 0.506 mmol), 3-iodopyridine (106 mg, 1.0 equiv), toluene (3.7 mL), $Pd(PPh_3)_4$ (7.7 mg, 10 mol %), and copper iodide (19.4 mg, 20 mol %) was heated to reflux for 1 day. The reaction mixture was then cooled, diluted with ethyl acetate, washed with sodium bicarbonate solution, and dried ($MgSO_4$) and the solvent was removed in vacuo to yield the crude product, which was purified using flash chromatography to yield the title compound (**46**) (8 mg, 5%). Mp 206−207 °C; ^{1}H NMR ($CDCl_3$) δ 1.99 (m, 4H, 2 × CH_2), 2.63 (m, 6H, 3 × CH_2), 2.74 (m, 2H, CH_2), 3.13 (m, 2H, CH_2), 3.45 (m, 4H, CH_2), 4.29 (m, 2H, CH_2), 6.87 (m, 1H,), 7.00 (m, 2H, ArH), 7.32 (m, 1H, ArH), 8.59 (dd, 1H, J = 4.8 and 1.7 Hz, ArH), 8.71 (m, 1H, ArH), 9.61 (s, 1H, ArH); MS *m*/*z* 500.4 $(M + H)^+$.

4-(4-Phenethylpiperazin-1-yl)-2-phenyl-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (44). Mp 271−272 °C; ^{1}H NMR ($CDCl_3$) δ 2.15−1.95 (m, 4H, 2 × CH_2), 2.75 (m, 6H, 3 × CH_2), 2.88 (m, 2H, CH_2), 3.21 (m, 2H, CH_2), 3.51 (m, 4H, 2 × CH_2), 4.34 (m, 2H, CH_2), 7.47−7.22 (m, 8H, 8 × ArH), 8.55 (m, 2H, 2 × ArH); MS *m*/*z* 463 $(M + H)^+$.

***N*-{4-[9-Cyano-4-(4-phenethylpiperazin-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluoren-2-yl]phenyl}acetamide (45).** Mp 281−283 °C; ^{1}H NMR (DMSO-d_6) δ 1.90 (m, 4H, 2 × CH_2), 2.09 (s, 3H, CH_3), 2.60 (m, 2H, CH_2), 2.64 (m, 4H, 2 × CH_2), 2.75 (m, 2H, CH_2), 3.08 (m, 2H, CH_2), 3.39 (m, 4H, 2 × CH_2), 4.33 (m, 2H, CH_2), 7.30−7.14 (m, 5H, 5 × ArH), 7.69 (m, 2H, 2 × ArH), 8.32 (m, 2H, 2 × ArH), 10.12 (br s, 1H, NH); MS *m*/*z* 520 $(M + H)^+$.

4-{4-[2-(3,4-Difluorophenyl)ethyl]piperazin-1-yl}-2-pyridin-4-yl-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (47). Mp 205−207 °C; ^{1}H NMR (DMSO-d_6) δ 3.48−2.92 (m, 14H, 7 × CH_2), 3.88 (m, 4H, 2 × CH_2), 4.26 (2H, m, CH_2), 6.99 (m, 1H, ArH), 7.24 (m, 3H, 3 × ArH), 8.42 (m, 2H, 2 × ArH), 8.73 (m, 2H, ArH), 10.94 (m, 1H, ArH); MS *m*/*z* 500 $(M + H)^+$.

4-{4-[2-(3,4-Difluorophenyl)ethyl]piperazin-1-yl}-2-*o*-tolyl-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (48). ^{1}H NMR ($CDCl_3$) δ 2.00−2.18 (m, 4H, 2 × CH_2), 2.62−2.90 (m, 11H, 4 × CH_2, CH_3), 3.26 (t, 2H, J = 6.2 Hz, CH_2), 3.48 (m, 4H, 2 × CH_2), 4.42 (t, 2H, J = 5.4 Hz, CH_2), 6.90−7.40 (m, 6H, 6 × ArH), 8.03 (m, 1H,ArH); MS *m*/*z* 513 $(M + H)^+$.

General Procedure for the Synthesis of Compounds 49 and 50 Is Exemplified by That for 49. 1-[4-(4-Phenethylpiperazin-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluoren-9-yl]propan-1-one (49). To a solution of ethylmagnesium bromide (1.0 M solution in *tert*-butyl methyl ether, 3.0 equiv, 1.16 mmol, 1.16 mL) and dry tetrahydrofuran (0.5 mL) was added a solution of 4-(4-phenethylpiperazine-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (150 mg, 0.39 mmol) in THF (2 mL) dropwise. The reaction mixture was heated to 60 °C for 5 h. The reaction mixture was then cooled, quenched with water, extracted into ethyl acetate, and dried ($MgSO_4$), and the solvent was removed in vacuo. The residue was purified using flash chromatography to yield the title compound as a white solid (**49**) (43.5 mg, 27%). Mp 129−130 °C; ^{1}H NMR ($CDCl_3$) δ 1.22 (t, 3H, J = 7.3 Hz, CH_3), 1.92−2.05 (m, 4H, 2 × CH_2), 2.65−2.75 (m, 6H, 3 × CH_2), 2.82−2.88 (m, 2H, CH_2), 3.33−3.40 (m, 6H, 3 × CH_2), 3.43 (t, 2H, J = 6.6 Hz, CH_2), 4.45 (t, 2H, J = 5.0 H, CH_2), 7.16−7.32 (m, 5H, 5 × ArH), 8.65 (s, 1H, ArH); MS *m*/*z* 418 (M + H). Anal. ($C_{25}H_{31}N_5O$) C, H, N.

[4-(4-Phenethylpiperazin-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluoren-9-yl]-phenylmethanone (50). Mp 140−142 °C; ^{1}H NMR ($CDCl_3$) δ 1.95−2.02 (m, 2H, CH_2), 2.03−2.12 (m, 2H, CH_2), 2.68−2.78 (m, 6H, 3 × CH_2), 2.85−2.90 (m, 2H, CH_2), 3.37 (t, 2H, J = 6.7 Hz, CH_2), 3.40−3.46 (m, 4H, 2 × CH_2), 4.46 (t, 2H, J = 5.6 Hz, CH_2), 7.20−7.28 (m, 3H, 3 × ArH), 7.30 (t, 2H, J = 7.4 Hz, 2 × ArH), 7.44 (t, 2H, J = 7.4 Hz, 2 × ArH), 7.54 (t, 1H, J = 7.4 Hz, ArH), 7.89 (d, 2H, J = 7.1 Hz, 2 × ArH), 8.51 (s, 1H, ArH); MS *m*/*z* 466 $(M + H)^+$.

4-(4-Phenethylpiperazin-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carboxylic Acid Methyl Ester (51). A mixture of 4-(4-phenethylpiperazin-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carboxylic acid amide (300 mg, 0.74 mmol) [prepared as for compound **55**], acetyl chloride (0.75 mL), and methanol (4 mL) was heated to 50 °C for 4 days. The solvent was then removed in vacuo, the residue was dissolved in dichloromethane, washed with sodium bicarbonate solution, and dried ($MgSO_4$), and the solvent was removed in vacuo to yield a solid, which was purified using flash chromatography to yield the title compound as an off-white solid (36 mg, 12%). Mp 154−155 °C; ^{1}H NMR ($CDCl_3$) δ 1.90−2.00 (m, 4H, 2 × CH_2), 2.60−2.70 (m, 6H, 3 × CH_2), 2.78−2.82 (m, 2H, CH_2), 3.29−3.38 (m, 6H, 3 × CH_2), 3.91 (s, 3H, CH_3), 4.35 (t, 2H, J = 5.8 Hz, CH_2), 7.12−7.25 (m, 5H, 5 × ArH), 8.68 (s, 1H, ArH); MS *m*/*z* 420 $(M + H)^+$; HPLC 97.64%.

9-(4,5-Dimethylthiazol-2-yl)-4-(4-phenethylpiperazin-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene (52). To a solution of 4-(4-phenethylpiperazine-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (**30**) (3.3 g, 8.55 mmol) in dimethylformamide (30 mL) was added sodium hydrosulfide hydrate (4.7 g, 42.73 mmol). The reaction mixture was heated at 60 °C for 5 days. After cooling, the reaction mixture was extracted into ethyl acetate, washed with water, and dried ($MgSO_4$) and the solvent was removed in vacuo to yield the crude product, which was purified using flash chromatography to yield the intermediate 4-(4-phenethylpiperazin-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbothioic acid amide (2.20 g, 61%). A mixture of 4-(4-phenethylpiperazin-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbothioic acid amide (70 mg, 0.167 mmol), triethylamine (1.2 equiv, 0.2 mmol, 28μL), and 3-bromo-2-butanone (1.2 equiv, 0.2 mmol, 30.2 mg) was heated to 100 °C for 2.5 h. The volatiles were then removed in vacuo, and the residue was triturated with water and filtered to yield a solid, which was purified using flash chromatography (2% MeOH in DCM) to yield the title compound as a beige solid (39.2 mg, 49%). Mp 150−152 °C; ^{1}H NMR ($CDCl_3$) δ 1.98−2.10 (m, 4H, 2 × CH_2), 2.38 (s, 3H,CH_3), 2.41 (s, 3H, CH_3), 2.66−2.75 (m, 6H, 3 × CH_2), 2.82−2.90 (m, 2H, CH_2), 3.42−3.48 (m, 4H, 2 × CH_2), 3.54 (t, 2H, J = 6.3 Hz, CH_2), 4.35 (t, 2H, J = 5.1 Hz, CH_2), 7.20−7.32 (m, 5H, 5 × ArH), 8.71 (s, 1H, ArH); MS *m*/*z* 473 $(M + H)^+$. Anal. ($C_{27}H_{32}N_6S$) C, H, N.

9-(5-Methyl[1,2,4]oxadiazol-3-yl)-4-(4-phenethylpiperazin-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene (53). A mixture of 4-(4-phenethylpiperazine-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (725 mg, 1.7 mmol), potassium carbonate (1.04 g, 7.5 mmol), and hydroxylamine hydrochloride (457 mg, 6.6 mmol) in ethanol (16 mL) was heated to reflux overnight. The reaction mixture was reduced in vacuo and triturated with water, and a precipitate was collected. This was triturated with hot dichloromethane to yield the desired amidoxime (**19**) (430 mg, 60%). A mixture of the above amidoxime (188 mg, 0.44 mmol) and acetyl chloride (1.5 equiv, 48 μL, 0.683 mmol) in pyridine (1.5 mL) was heated to 60 °C for 6 h. The reaction mixture was cooled, extracted into dichloromethane, washed with sodium bicarbonate solution, and dried ($MgSO_4$) and the solvent was removed in vacuo to yield the crude product, which was purified by flash chromatography to yield the title compound as a beige solid (37 mg, 5%). Mp 79 °C; ^{1}H NMR ($CDCl_3$) δ 2.00−2.14 (m, 4H, 2 × CH_2), 2.70 (s, 3H, CH_3), 2.70−2.80 (m, 6H, 2 × CH_2), 2.83−2.92 (m, 2H, CH_2), 3.40−3.52 (m, 6H, 3 × CH_2), 4.40 (t, 2H, J = 6.0 Hz, CH_2), 7.20−7.33 (m, 5H, 5 × ArH), 8.77 (s, 1H, ArH); MS m/z 444 $(M + H)^+$.

4-(4-Phenethylpiperazin-1-yl)-9-pyrimidin-2-yl-5,6,7,8-tetrahydro-1,3,4b-triazafluorene (54). To *N*-hydroxy-4-(4-phenethylpiperazin-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carboxamidine (**19**) (430 mg, 1.02 mmol) in acetic acid (5 mL) was added acetic anhydride (1.6 equiv, 154 μL, 1.63 mmol), and the mixture stirred for 10 min. Palladium (10%) on carbon was then added (110 mg), and the mixture was stirred under a positive pressure of hydrogen for 5 h. The reaction mixture was then filtered through Celite, and the volatiles were removed in vacuo. The residue was dissolved in dichloromethane, washed with sodium bicarbonate solution, and dried ($MgSO_4$) and the solvent was removed in vacuo to yield the desired amidine (**21**) (382 mg, 95%). This was converted into the dihydrochloride salt by treatment with HCl in methanol.

To 4-(4-phenethylpiperazin-1-yl)-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carboxamidine dihydrochloride salt (**21**) (105 mg, 0.22 mmol) in Dowtherm (2 mL) was added potassium carbonate (30.4 mg, 0.44 mmol) and malonaldehyde bis-(dimethylacetal) (120 μL, 0.265 mmol). The reaction mixture was heated to 175 °C for 5 h. The reaction mixture was cooled, diluted with ethyl acetate, extracted into 2 M HCl, neutralized ($NaHCO_3$), extracted into dichloromethane, and dried ($MgSO_4$) and the solvent was removed in vacuo to yield crude product, which was purified using flash chromatography to yield the title compound (80 mg, 83%). Mp 164 °C; ^{1}H NMR ($CDCl_3$) δ 2.05−2.20 (m, 4H, 2 × CH_2), 2.78−2.90 (m, 6H, 3 × CH_2), 2.93−2.98 (m, 2H, CH_2), 3.50 (m br, 4H, 2 × CH_2), 3.60 (t, 2H, J = 6.7 Hz, CH_2), 4.50 (t, 2H, J = 5.8 Hz, CH_2), 7.15 (t, 1H, J = 4.8 Hz, CH_2), 7.28−7.40 (m, 5H, 5 × ArH), 8.88 (s, 1H, ArH), 8.95 (d, 2H, J = 4.8 Hz, 2 × ArH); MS m/z 440 $(M + H)^+$.

4-{4-[2-(3,4-Difluorophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carboxylic Acid Amide (55). A solution of KOH (465 mg, 8.3 mmol) in water (2 mL) was added to a suspension of 4-{4-[2-(3,4-difluorophenyl)ethyl]-piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carbonitrile (500 mg, 1.1 mmol) in ethanol (6 mL). The reaction mixture was stirred for 6 h at reflux and then cooled. The precipitate was collected by filtration and washed with ethanol to yield the title compound as a white solid (179 mg, 37%). Mp 257−259 °C; ^{1}H NMR ($CDCl_3$) δ 2.00−2.15 (m, 4H, 2 × CH_2), 2.66−2.82 (m, 6H, 3 × CH_2), 2.84−2.88 (m, 2H, CH_2), 3.48 (m br, 4H, 2 × CH_2), 3.59 (t, 2H, J = 6.2 Hz, CH_2), 4.43 (t, 2H, J = 5.9 Hz, CH_2), 5.52 (s br, 1H, NH), 6.99−7.02 (m, 1H, ArH), 7.08−7.17 (m, 2H, ArH), 8.63 (s, 1H, ArH), 8.84 (s br, 1H, NH); MS m/z 441 $(M + H)^+$; HPLC 95.23%.

General Procedure for the Synthesis of Compounds 56−58 Is Exemplified by That for 57. 4-{4-[2-(3,4-Difluorophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carboxylic Acid 2,3-Dimethoxybenzylamide (57).[25a] To 4-{4-[2-(3,4-difluorophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carboxylic acid amide (**55**) (62 mg, 0.14 mmol) in acetonitrile (1.5 mL) was added 2,3-dimethoxybenzaldehyde (70 mg, 0.42 mmol), triethylsilane (75 μL, 0.42 mmol), and trifluoroacetic acid (51 μL, 0.546 mmol). The reaction mixture was heated to reflux for 18 h. The solvent was then removed in vacuo, the residue was dissolved in ethyl acetate, washed with sodium bicarbonate solution, and dried ($MgSO_4$), and the solvent was removed in vacuo to yield the crude product. This was recrystallized from dichloromethane/hexane to yield the desired title compound (76 mg, 92%). Mp 177−178 °C; ^{1}H NMR ($CDCl_3$) δ 1.95−2.01 (m, 4H, 2 × CH_2), 2.60−2.72 (m, 6H, 3 × CH_2), 2.78−2.82 (m, 2H, CH_2), 3.36 (br, 4H, 2 × CH_2), 3.55 (t, 2H, J = 6.3 Hz, CH_2), 3.83 (s, 3H, CH_3), 3.90 (s, 3H, CH_3), 4.30 (t, 2H, J = 5.8 Hz, CH_2), 4.72 (d, 2H, J = 6.0 Hz, CH_2), 6.80 (d, 1H, J = 6.0 Hz, ArH), 6.84−6.86 (m, 1H, ArH), 6.98−7.10 (m, 4H, ArH), 8.50 (s, 1H, ArH), 9.35 (t, 1H, ArH); MS m/z 591 $(M + H)^+$; HPLC 96.25%.

4-{4-[2-(3,4-Difluorophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carboxylic Acid Ethylamide (56). Mp 162−163 °C; ^{1}H NMR ($CDCl_3$) δ 1.44 (t, 3H, J = 7.2 Hz, CH_3), 2.08−2.18 (m, 4H, 2 × CH_2), 2.77−2.90 (m, 6H, 3 × CH_2), 2.92−2.98 (m, 2H, CH_2), 3.52−3.60 (m, 4H, 2 × CH_2), 3.65−3.73 (m, 4H, 2 × CH_2), 4.50 (t, 2H, J = 6.0 Hz, CH_2), 7.02−7.08 (m, 1H, ArH), 7.14−7.28 (m, 2H, 2 × ArH), 8.70 (s, 1H, ArH), 9.05 (br s, 1H, NH); MS m/z 469 $(M + H)^+$.

4-{4-[2-(3,4-Difluorophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carboxylic Acid (Pyridin-2-ylmethyl)amide (58). Mp 147−149 °C; ^{1}H NMR ($CDCl_3$) δ 1.88−2.00 (m, 4H, 2 × CH_2), 2.08−2.17 (m, 6H, 3 × CH_2), 2.20−2.26 (m, 2H, CH_2), 3.32−3.38 (m, 4H, 2 × CH_2), 3.48 (t, 2H, J = 6.4 Hz, CH_2), 4.28 (t, 2H, J = 6.0 Hz, CH_2), 4.78 (2H, d, J = 5.9 Hz, CH_2), 6.82−6.86 (m, 1H, ArH), 6.93−7.04 (m, 2H, ArH), 7.10 (t, 1H, J = 7.6 Hz, ArH), 7.30 (d, 1H, J = 7.6 Hz, ArH), 7.56 (t, 1H, J = 7.6 Hz, ArH), 8.50 (s, 1H, ArH), 8.50−8.55 (d, 1H, J = 7.6 Hz, ArH), 9.56 (m, 1H, CONH); MS m/z 532 $(M + H)^+$. Anal. ($C_{29}H_{31}F_2N_7O$) C, H, N.

4-{4-[2-(3,4-Difluorophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carboxylic Acid Pyridin-3-ylamide (59).[25b] A mixture of 4-{4-[2-(3,4-difluorophenyl)ethyl]piperazin-1-yl}-5,6,7,8-tetrahydro-1,3,4b-triazafluorene-9-carboxylic acid amide (**55**) (100 mg, 0.227 mmol) and 3-bromopyridine (1.1 equiv, 0.25 mmol) was stirred in 1,4-dioxane (1.5 mL). To this was added cesium carbonate (1.5 equiv, 111 mg), tris-(dibenzylideneacetone)dipalladium (2 mg), and xanthphos (2 mg). The reaction mixture was heated to 100 °C overnight. After cooling, the reaction mixture was extracted into dichloromethane, washed with water, and dried ($MgSO_4$) and the solvent was removed in vacuo to yield the crude product, which was purified using flash chromatography to yield the title compound (5 mg, 4%). Mp 227−228 °C; ^{1}H NMR ($CDCl_3$) δ 1.90−2.03 (m, 4H, 2 × CH_2), 2.60−2.71 (m, 6H, 3 × CH_2), 2.72−2.78 (m, 2H, CH_2), 3.38−3.43 (m, 4H, 2 × CH_2), 3.55 (t, 2H, J = 6.0 Hz, CH_2), 4.32 (t, 2H, J = 6.0 Hz, CH_2), 6.82−6.88 (m, 1H, ArH), 6.95−7.04 (m, 2H, ArH), 7.22−7.25 (m, 1H, ArH), 8.23−8.28 (m, 2H, ArH), 8.55 (s, 1H, ArH), 8.80 (s, 1H, ArH); MS m/z 518 $(M + H)^+$.

Biology Assays. Drug Accumulation Assay (MRP). Compounds were assayed for inhibition of MRP1-dependent transport of the radiolabeled cytotoxic agent and MRP substrate, daunomycin. 1×10^4 COR.L23/R cells (MRP expressing human non-small-cell lung carcinoma MDR subline) were seeded 48 h prior to assay into 96-well opaque culture plates. Compounds were serially diluted over a range of concentrations from 100 to 0.015 μM in assay medium containing tritiated daunomycin at 0.3 μCi/mL and incubated with COR.L23/R cells at 37 °C for 2 h before washing and determination of cell-associated radioactivity. Results are expressed as an IC_{50} for daunomycin accumulation, where 100% accumulation is that observed in the presence of the known MRP1 modulator verapamil[30] at 100 μM.

Potentiation Assay. The ability of modulators to potentiate the cytotoxicity of doxorubicin was evaluated in the COR.L23/R cell line as outlined previously.[26] IC_{50} values for doxo-

rubicin (concentration resulting in 50% inhibition of cell growth) were calculated from plotted results using untreated cells as 100%. EC_{50} values for modulators (concentration required to give 50% of full reversal) were obtained from graphs of potentiation index (ratio of IC_{50} of cytotoxic drug alone to IC_{50} of cytotoxic drug in the presence of modulator) plotted against concentration of modulator.

Similar protocols[26] were applied for Pgp assays with the use of Pgp expressing murine mammary carcinoma EMT6/AR1.0 subline.

Inhibition of CYP 3A4 activity was measured by determining the conversion of testosterone to 6β-hydroxytestosterone in the presence and absence of modulators according to a literature protocol.[31] Briefly, modulators (0.1−100 μM) were incubated at 37 °C with human liver microsomes (0.1 mg/mL) in the presence of testosterone (110 μM). The reaction was initiated by the addition of a NADPH generating system and stopped by addition of methanol after 10 min. Supernatants were analyzed by HPLC using an acetonitrile/water gradient containing 0.05% orthophosphoric acid on an Intersil ODS-2 column (4.6 mm × 250 mm, 5 μm) with UV detection at 300 nm. IC_{50} (concentration resulting in 50% inhibition) values were calculated from graphs of percent control activity versus drug concentration.

Pharmacokinetic Studies. All animal experimentation was performed to U.K. Home Office regulations, and the UKKCCCR guidelines were adhered to throughout the studies. Compounds were administered intravenously, intraperitoneally , or orally at 20−50 mg/kg to female Balb/c mice (three to four animals per time point). Blood samples were collected from anesthetized animals by cardiac puncture at various times between 0.03 and 24 h and centrifuged to prepare plasma, which was stored at −40 °C until analysis. Samples (100 μL) were extracted with methanol (300 μL) at −40 °C (15 min incubation), and the supernatants were analyzed by HPLC as outlined above. The area under the concentration−time curves (AUC) and elimination half-life ($t_{1/2}$) of the compounds were calculated using noncompartmental analysis using PCModfit (Gamms Ltd., Hertfordshire, U.K.). AUC values were calculated using the linear trapezoidal rule up to the peak concentration and the logarithmic trapezoidal rule for postpeak concentration using PCModfit.

In Vivo Efficacy Studies. The efficacy of compounds (modulators) was evaluated using the resistant COR L23/R cell xenografts. Female CD1 athymic mice were inoculated subcutaneously with 1×10^6 COR/L23R cells in 100 μL of phosphate buffered saline. When the tumors reached a mean diameter of 0.4−0.6 cm, the animals were randomized into groups of six and treated with vincristine (iv) or modulator (ip or po) alone or in combination on days 0 (start of treatment) and 5. The modulators were administered once before (typically at −30 min) and once after (typically at 4 h) the cytotoxic drug administration. Control animals were treated with vehicle alone. Tumor volumes and body weights were measured at least three times per week as outlined previously.[32] The mean relative tumor volume was calculated using the tumor volume on the first day of treatment (day 0). The ratio (mean relative tumor volume of the treated group)/(the mean relative tumor volume of control group) × 100 (T/C, %) was calculated each time the tumors were measured. Statistical analysis was performed using two-way ANOVA with Bonferroni posttests.

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Note Added after ASAP Posting. This manuscript was released ASAP on 2/7/2004 with an incorrect reference citation three lines below structures **1** and **2**. The correct version was posted on 2/10/2004.

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