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Bioorganic & Medicinal Chemistry 13 (2005) 2723–2739
Bioorganic & Medicinal Chemistry

Design, synthesis and characterization of a novel class of coumarin-based inhibitors of inducible nitric oxide synthase

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Received 28 October 2004; revised 17 February 2005; accepted 17 February 2005

Abstract—Inducible nitric oxide synthase (iNOS) has been implicated in various central and peripheral pathophysiological diseases. Our high throughput screening initially identified a weak inhibitor of iNOS, thiocoumarin **13**. From this lead, a number of potent derivatives were prepared that demonstrate favorable potency, selectivity and kinetics. Compound **30** has an IC_{50} of 60 nM for mouse iNOS and 185-fold and 9-fold selectivity for bovine eNOS and rat nNOS, respectively. In cellular assays for iNOS, this compound has micromolar potency. Furthermore, two compounds (**16** and **30**) demonstrate a reasonable pharmacokinetic profile in rodents. The synthesis, SAR, and biological activity of this novel class of compounds is described.

1. Introduction

Nitric oxide (NO) is a molecular messenger that is synthesized by nitric oxide synthase (NOS) enzymes. NO is implicated in a variety of physiological and pathological states, both in the central and peripheral nervous systems. In the central nervous system (CNS), activated parenchymal and perivascular microglia, astrocytes, neurons, and even endothelial cells (after activation by proinflammatory cytokines) produce NO. The potential for pathological consequences as a result of NO overproduction is significant in the CNS. The reaction of NO with super oxide anion (O_2^-), another free radical produced by activated macrophages, forms peroxynitrite ($ONOO^-$). Peroxynitrite is a footprint of NO production and is a strong nitrating agent capable of producing nitrotyrosines (NT).[1]

The reaction which produces NT may also cause alteration of tyrosine phosphorylation dependent signaling, modification of protein conformation by introduction of a negative charge, and initiation of autoimmune processes because nitrophenols are known to be highly antigenic. Myelin protein can be one such target in the CNS for N-nitrosylation. All these effects of NO may account for the oligodendrocyte loss that is observed in multiple sclerosis (MS). In addition to its cytotoxic actions, NO can also block electrical conduction in demyelinated and newly remyelinated axons.[2] Under ischemic conditions, cessation of blood flow to the brain sets in motion a cascade of events resulting in neuronal cell death and enhanced production of NO. The reactive product of NO ($ONOO^-$) has been proposed to be an important mediator.[1,3]

Nitric oxide synthase carries out the oxidation of the guanidine moiety of L-arginine to deliver citrulline and NO (Scheme 1). Three quite distinct isoforms of NOS, the enzymes responsible for the production of NO, have been identified that are capable of generating NO in a wide variety of tissues.[4] These isoforms are nNOS (type I or NOS-1) being the isoform first found (and predominating) in neuronal tissue, iNOS (type II or NOS-2) being the isoform, which is inducible in a wide range of cells, and eNOS (type III or NOS-3) being the

Keywords: iNOS inhibition; Coumarin.
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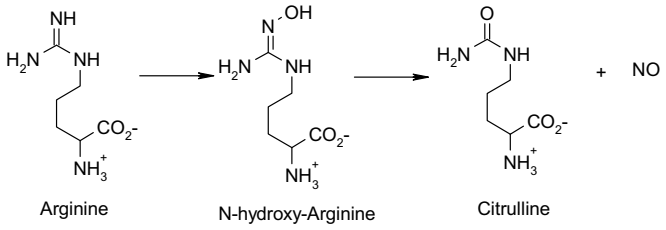
doi:10.1016/j.bmc.2005.02.036



Scheme 1. NOS oxidation of arginine.

isoform first found in vascular endothelial cells. These isoforms are also differentiated based on their constitutive (eNOS and nNOS) versus inducible (iNOS) expression, and their calcium dependence (eNOS and nNOS) or independence (iNOS). The importance of iNOS and NO in MS was highlighted by clinical studies that demonstrated increased iNOS mRNA in the brain,[5,6] increased NT staining in MS tissue, and NO metabolites in the spinal fluid.[7] Clarification of the role of iNOS in stroke was provided by the observation that mice lacking the gene for iNOS have significantly reduced infarct volumes compared with wild-type controls.[8] Also, iNOS mRNA was increased in transient cerebral ischemia in rats and the expression was mainly localized in vascular cells of the infarcted region.[9]

Initially, the best-known inhibitors of iNOS were analogs of L-arginine (Fig. 1) such as N^G-methyl-L-arginine (L-NMA) **1**,[10,11] N^G-nitro-L-arginine (L-NNA) **2**,[12] *N*-iminoethyl-L-ornithine (L-NIO) **3**,[13] L-thiocitrulline **4**,[14] and *N*-iminoethyl-L-lysine (L-NIL) **5**.[15] All lack selectivity over other NOS isoforms and therefore result in undesirable pharmacological effects in vivo. As such, most amidine or guanidine moiety containing compounds were not particularly selective for iNOS relative to the other isoforms. Based on the structure of L-NIL, acetamidine derivatives such as *S*-[2-[(1-iminoethyl)amino]ethyl]-4,4-dioxo-L-cysteine (GW273629) **6** and *S*-[2-[(1-iminoethyl)amino]ethyl]-L-homocysteine (GW274150) **7** were designed. However, like L-NIL, these were time-dependent inhibitors of iNOS.[4] This is undesirable because these inhibitors could lead to irreversible inhibition of iNOS, the physiological consequences of which are unknown. AstraZeneca researchers reported good activity of 3,4-dihydro-1-isoquinolinamines **8** (Fig. 2) against iNOS, but poor cellular potency and recently found 2-aminopyridine derivatives as highly selective inhibitors of iNOS.[16,17] Replacement of the benzo ring with a thieno fused ring resulted in thienopyridines **9** that showed potent activity against iNOS but the best compound produced hypotension in vivo.[18] Tinker et al. demonstrated potency, selectivity and efficacy in the rat adjuvant-induced arthritis model for two representative compounds (AR-C85016 **10** and AR-C102222 **11**) from the 1,2-dihydro-4-quinazolinamine series.[19] Synthesis of dihydropyrindin-2(1*H*)-imines and 1,5,6,7-tetrahydro-2*H*-azepin-2-imines **12** has been described, but these lacked suffi-

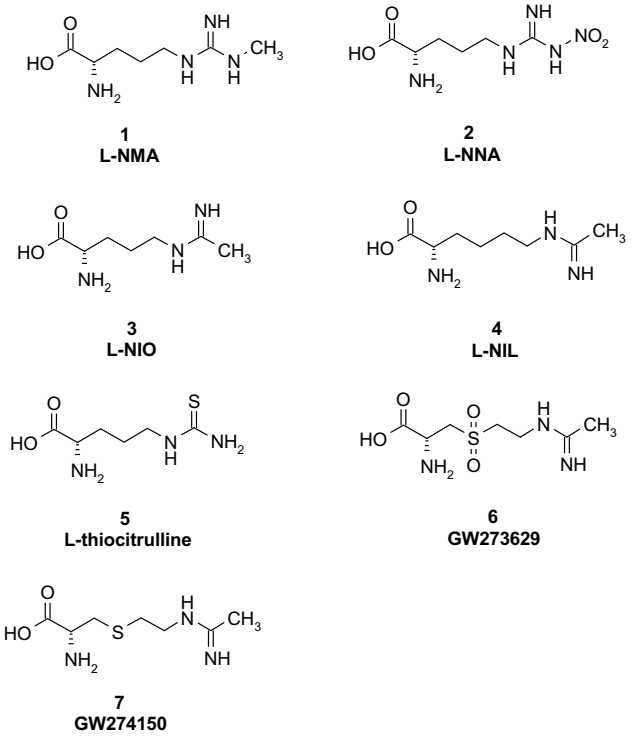


Figure 1. NOS inhibitors based on L-arginine.

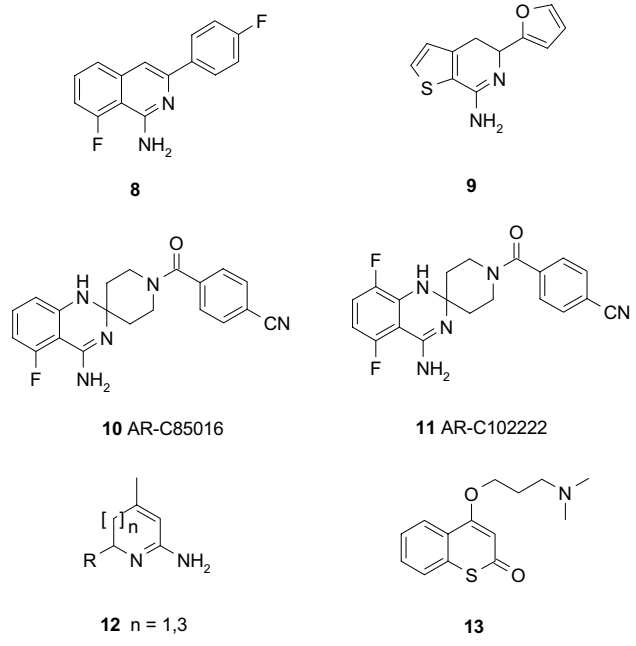
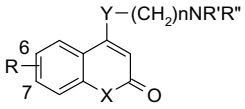


Figure 2. Amidine-type NOS inhibitors and thiocoumarin **13**.

Table 1. Inhibitory activity of compounds **13**–**21**



Compound	R	R′	R″	*n*	X	Y	iNOS IC_{50} (μM)
13	H	CH_3	CH_3	3	S	O	18 (h)
14	6-Cl	H	H	3	O	CH_2	3.57 (h)
15	6-Cl	H	H	3	NH	CH_2	2.15 (h)
16	6-Cl	H	H	3	NH	O	0.407 (m)
17	H	CH_3	CH_3	3	NH	O	2.63 (h), 10.4 (m)
18	H	H	H	3	NH	O	3.03 (h), 1.86 (m)
19	H	H	H	3	NCH_3	O	14% I @ 10 μM (m)
20	H	CH_3	CH_3	3	NCH_3	O	6.7% I @ 10 μM (m)
21	6-Cl	H	H	4	O	CH_2	2.6 (m)

h = human; m = mouse.

cient selectivity over eNOS (<20-fold) and selectivity over nNOS is unknown.[20]

Our goal was to discover small molecule inhibitors of iNOS with good brain penetration and with acceptable selectivity over the other isozymes. Screening efforts identified thiocoumarin **13** (Fig. 2) as a weak (IC_{50} = 18 μM) albeit interesting inhibitor of iNOS. The fact that it contained neither the amidine nor guanidine functionality, warranted further investigation and a limited SAR study was undertaken. This included the preparation of analogs (Tables 1 and 2 and Fig. 3) with various simple substitutions on the aromatic ring, investigation of the propyl-tethered amine and changes to the thiolactone functionality of **13**. Here we report on the design, synthesis and characterization of coumarin-based inhibitors of iNOS.

2. Chemistry

In undertaking the SAR study of the thiocomarin lead **13** an immediate improvement in activity as well as synthetic adaptability was found by replacement of the sulfur of **13** with oxygen. Thus, our efforts focused primarily on lactones (i.e., coumarins) and no further thio-containing analogs were prepared. A Mitsunobu protocol[21] or the displacement of an alkyl halide or mesylate by an appropriate hydroxy-substituted intermediate[22,23] were the two methods utilized to prepare essentially all of the compounds (Scheme 2).

Where a tethered primary amine was the product (e.g., compounds **14**, **16**, Table 1) a protecting group was required in the alkylation step. These could be further derivatized after deprotection in a straightforward manner (e.g., acylation to deliver amides **26** and **49** and guanylation[24] to afford **27**, **45**, and **47**, Table 2). Mesylation of the primary amine (compound **25**) as well as isothiourea formation (compounds **46** and **48**) were accomplished using standard methods. Aminoquinolines **61** and **62** (i.e., the introduction of an amidine-type functionality ubiquitous in the NOS inhibitors mentioned earlier, Scheme 2), and quinolones **16**–**20** (Table 1), were synthesized in a manner analogous to their respective oxygen counterparts. The all-carbon tether compounds **14**, **15** and **21** (Table 1) were prepared utilizing the organo-zinc cross-coupling reaction of Yang and co-workers, which was optimized using $Pd(PPh_3)_2Cl_2$ as catalyst (Scheme 3).[25,26]

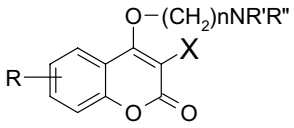
3. Results and discussion

As can been seen from the summary of the SAR in Table 3 a lactone or lactam functionality was preferable to the thiolactone with the former delivering the most potency. Variation of the propyl tether included chain extension and contraction (Tables 1 and 2) as well as attempts to restrict degrees of freedom with cyclic structures (compounds **53**–**60**, Fig. 3). Acylation of the tethered amine led to a significant loss of activity. Thus, maintaining a single basic nitrogen was important with the primary substituted aminopropyl functionality preferred. Modeling predictions relative to aromatic substitution of the coumarin were borne out in the fact that substitution at the 6-position (compound **30**) was best with putative Van der Waals interaction between the 6-chloro of **30** and the side chain (the phenyl ring) of phenylalanine 363 (F363) in the enzyme (Fig. 4).

This type of interaction was first discovered in previous factor Xa work, which led to a breakthrough in trypsin-like serine protease inhibitor design.[27,28] The substitution at the 4-position (the aminopropyl group) enables H-bonding interactions with the two carboxylic acids of HEME, which led us to believe that it is important to preserve the hydrogens of the terminal amino group. It is rather difficult to understand the role of oxygen in the coumarin ring, especially due to its close proximity to the HEME ring underneath and the backbone oxygen of tryptophan 366 (W366). It is our best understanding so far that the oxygen of W366 prefers a H-bonding group nearby and that the distance in between may favor a water-mediated H-bond more than a direct one.

The best compound, **30**, was evaluated further to examine the kinetics of iNOS inhibition using the human

Table 2. Inhibitory activity for coumarin derivatives



Compound	R	R′	R″	*n*	X	iNOS IC_{50} (μM)
22	H	H	H	2	H	>10 (h), 73.0 (m)
23	6-Cl	H	H	2	H	10.3 (m)
24	H	H	CH_3	2	H	>10 (h,m)
25	6-Cl	H	SO_2CH_3	3	H	15.3 (m)
26	H	H	$COCH_3$	2	H	nt
27	H	H	$(C{=}NH)NH_2$	2	H	23.5 (h), 10.1 (m)
28	6,7-Di-CH_3	H	H	3	H	<5% I @ 50 μM (m)
29	7-OCH_3	H	H	3	H	24% I @ 50 μM (m)
30	6-Cl	H	H	3	H	0.095 (h), 0.061 (m)
31	6-Br	H	H	3	H	0.102 (h), 0.143 (m)
32	6-F	H	H	3	H	1.11 (h), 0.441 (m)
33	6-OCH_3	H	H	3	H	31% I @ 50 μM (m)
34	6-CH_3	H	H	3	H	0.296 (h), 0.304 (m)
35	6,8-Di-Br	H	H	3	H	<5% I @ 50 μM (m)
36	5-Cl	H	H	3	H	23% I @ 50 μM (m)
37	H	H	H	3	H	7.58 (h), 11.9 (m)
38	H	H	CH_3	3	H	>10 (h), 17.8 (m)
39	6-Cl	H	CH_3	3	H	0.111 (h), 0.246 (m)
40	H	CH_3	CH_3	3	H	4.0 (h)
41	7-OCH_3	CH_3	CH_3	3	H	24.3% I @ 10 μM (m)
42	6-Cl	CH_3	CH_3	3	H	4.1 (h), 1.2 (m)
43	6-CH_3	CH_3	CH_3	3	H	4.7 (h), 3.0 (m)
44	6,7-Di-CH_3	CH_3	CH_3	3	H	13.4% I @ 10 μM (m)
45	H	H	$(C{=}NH)NH_2$	3	H	16.5 (h), 9.6 (m)
46	H	H	$(C{=}S)NHCH_3$	3	H	0% I @ 10 μM (m)
47	6-Cl	H	$(C{=}NH)NH_2$	3	H	0.509 (h), 0.388 (m)
48	6-Cl	H	$(C{=}S)NHCH_3$	3	H	20% I @ 10 μM (m)
49	6-Cl	H	$COCH_3$	3	H	8.1% I @ 10 μM (m)
50	5-Cl	H	H	4	H	0.2% I @ 10 μM (m)
51	6-Cl	H	H	4	H	1.98 (h), 1.01 (m)
52	6-Cl	H	H	5	H	1.33 (m)
63	6-Cl	H	H	3	CH_3	23% I @ 10 μM (m)

h = human; m = mouse; nt = not tested.

enzyme. Compound **30** inhibits human iNOS by competing with arginine at the substrate binding site with a K_i of 104 nM (Fig. 5). It was also shown that **30** was not a time-dependent inhibitor of human iNOS (Fig. 5). The enzyme does not appear to lose activity during the preincubation time as observed by approximately the same levels of product formation. This feature distinguishes **30** from other compounds described in the literature. Furthermore, inhibition of human iNOS by **30** was altered by ~2-fold in the presence of 10% FCS, indicating that protein binding was not significant (Fig. 5). Despite this observation, the activity in various cell types was not optimal (3–17 μM for primary cells vs 21 μM to >50 μM in other cells). To rule out a physical problem with cell penetration, we measured (by LCMS) the cell and medium concentration of compound **30** after centrifugation. The results (not shown) indicated that the cell pellet to medium ratio of compound was ~1. The ratio of 1 means that the compound does not have a physical problem per se of cell penetration. Although this fact does not explain the lack of cellular activity, a difference in mechanism or kinetics of inhibition may be contributing factors. Our compounds are reversible and time-independent inhibitors in contrast to others described in the literature and this may have influenced the cellular potency. In addition, we have not ruled out the possibility of compound metabolism in these specific cell types.

Two compounds, **16** and **30**, were selected for pharmacokinetic studies (Table 4). Compound **16**, the lactam analog of lactone **30**, has an IC_{50} = 407 nM against mouse iNOS. After intravenous dosing, the total plasma clearance (4.71 L/h/kg) of **16** approximated the hepatic blood flow (5.4 L/h/kg) in mice. Therefore it can be classified as a high clearance compound. The volume of distribution of **16** was extensive (10.2 L/kg), such that it surpassed the total body water (0.72 L/kg) in mouse. The high volume of distribution contributed to the relatively long half-life. Brain penetration was examined after intravenous dosing. The compound penetrated the brain tissue poorly and the extent was lower than that observed earlier for compound **30**.

Compound **30** has an IC_{50} = 60 nM against mouse iNOS with 185-fold selectivity over eNOS and 9-fold

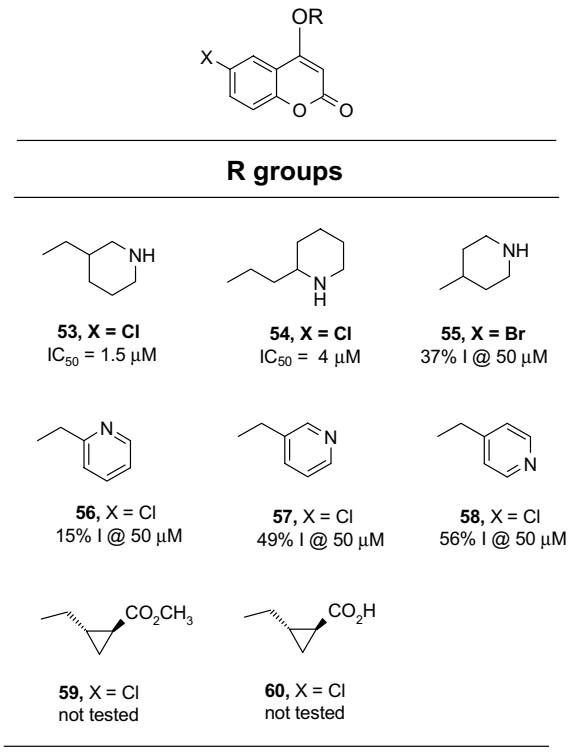
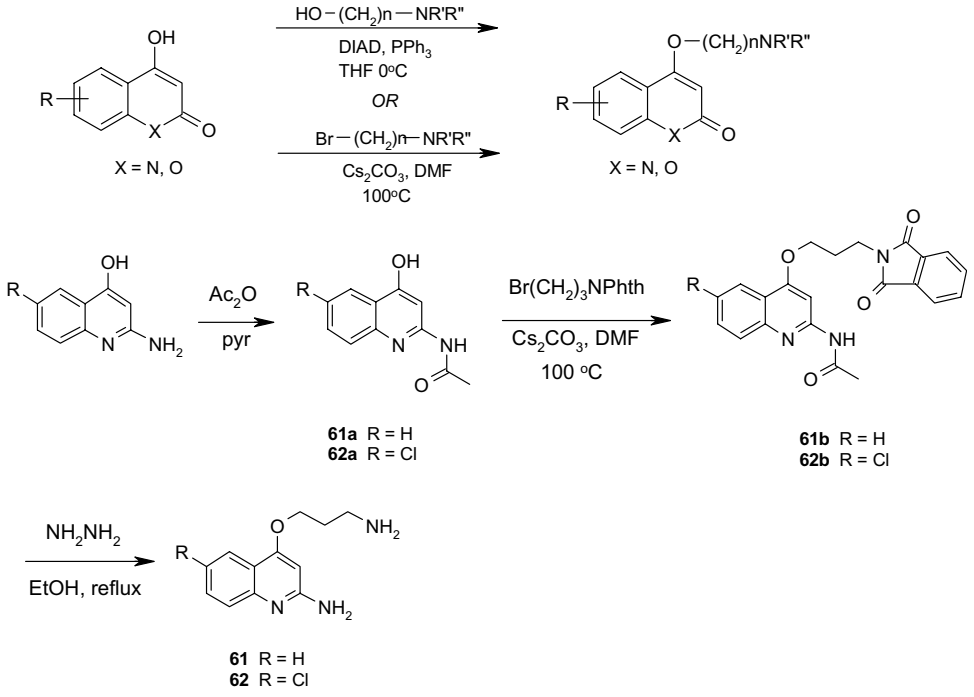


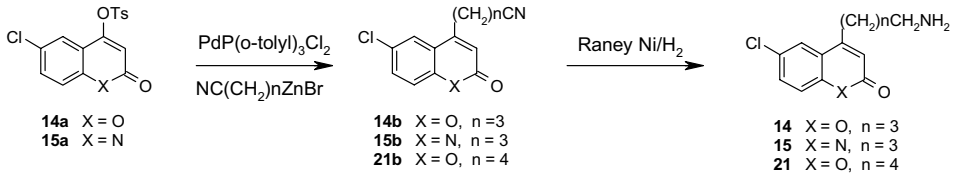
Figure 3. List of compounds prepared (continued). Activity and % inhibition are reported for mouse iNOS.

over nNOS. As shown in Table 4, coumarin **30** has a promising pharmacokinetic profile in mice and rats. After intravenous dosing, the total plasma clearance for **30** in mice (13.1 L/h/kg) and rats (25 L/h/kg) was more than the hepatic blood flow in those species (mice = 5.4 L/h/kg, rats = 3.31 L/h/kg). Therefore, **30** can be classified as a high clearance drug. The volume of distribution was extensive in both mice (3.58 L/kg) and rats (5.23 L/kg) in that it surpassed the total body water in both species (mice = 0.72 L/kg, rats = 0.67 L/kg). However, since the clearance was much higher than the volume of distribution, the half-life was short. Oral administration of the 10 mg/kg dose resulted in rapid absorption and absolute bioavailability was 31% and 40% in mice and rats, respectively. Brain penetration of **30** was excellent in mouse compared to that in rats. In mouse, the brain concentrations paralleled the plasma concentrations indicating good equilibrium between the two compartments.

A metabolic study was performed to specifically understand the reason for the high total clearance of **30** in vivo and to determine whether a major hepatic component was involved. After 1 h incubation, 99.7% and 12.9% of the parent was metabolized in human and mouse microsomes, respectively. In human microsomes, the metabolic pathway was independent of NADPH (same metabolites observed in microsomal incubations carried out in the absence of NADPH). In mouse microsomes, the metabolic pathway was NADPH dependent (no metabolites observed in mouse microsomal incubations carried out in the absence of NADPH). Although the metabolism profile was quantitatively different, qualitatively similar metabolites were observed in both human and mouse microsomes in the presence of NADPH. Several metabolites were observed in human and mouse microsomal incubations. The 4-hydroxy coumarin **64** (Scheme 4) (*m*/*z* 197, dealkylation of the ether

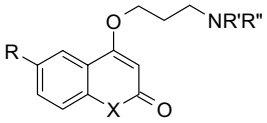


Scheme 2. Synthetic scheme for the preparation of coumarins and quinolines. (Aminoquinolines **61** and **62** had IC_{50}'s greater than 10 μM against mouse iNOS.)



Scheme 3. Synthesis of all-carbon tether compounds.

Table 3. SAR summary



Compd	X	R	R′	R″	Human iNOS IC_{50} (μM)	Mouse iNOS IC_{50} (μM)	Bovine eNOS IC_{50} (μM)	Rat nNOS IC_{50} (μM)	A172 cell IC_{50} (μM)	Raw cell IC_{50} (μM)
13	S	H	CH_3	CH_3	18	—	—	—	—	—
37	O	H	H	H	7.58	11.9	69.6	9.11	—	—
42	O	Cl	CH_3	CH_3	4.08	1.2	>100	7.96	>50	46
40	O	H	CH_3	CH_3	4.01	>10	>100	>10	—	—
17	NH	H	CH_3	CH_3	2.64	10.4	>100	>10	—	—
39	O	Cl	H	CH_3	0.11	0.25	>100	0.53	—	>50
16	NH	Cl	H	H	—	0.41	—	—	>50	>50
30[a]	O	Cl	H	H	0.09	0.06	11.1	0.56	>50	21

[a] IC_{50} in rat primary astrocytes and microglia was 17 and 3 μM, respectively.

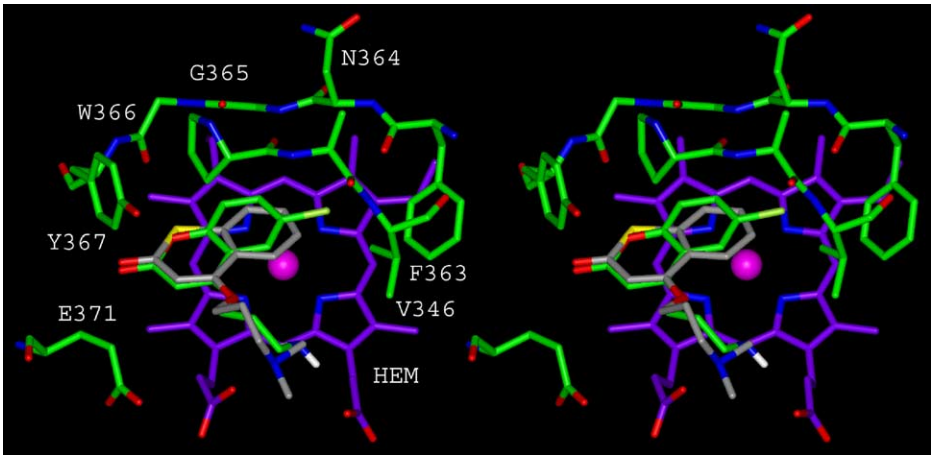


Figure 4. Stereo view of the iNOS binding pocket with thiocoumarin **13** (X-ray coordinates have been deposited in the Brookhaven Protein Databank with a pdb code of 2BHJ) and coumarin **30** (a computer model based on 2BHJ) superimposed. There are several key interactions between the inhibitors and the enzyme: (a) H-bonding/charge-charge interaction with backbone W366; (b) hydrophobic interaction with chlorine of the inhibitors and phenyl ring of F363; (c) H-bonding interaction between the amino group of the inhibitors and the carboxylic acid groups of HEME; (d) p stacking between the inhibitors and HEME ring.

moiety) was the major metabolite in mouse, whereas human microsomes indicated the others in significant amounts. In conclusion, the human microsomal incubations of **30** carried out in the absence of NADPH indicate the involvement of a CYP P450 independent pathway.

The synthesis of the all-carbon tether compounds **14**, **15**, and **21** were attempts at thwarting this metabolic pathway since the hydrolysis to 4-hydroxycoumarin **64** is not possible. Unfortunately, these derivatives did not improve the ADME properties of **30**. They were much less active in the iNOS inhibition assay even though no major geometric changes to the molecule (O and C both sp^3 hybridized) were apparent. Another strategy involved the introduction of a methyl group into the 3 position (Table 2, compound **63**). Since it is known that warfarin-like oral anticoagulants (3-substituted hydroxycoumarins) have long half-lives and low clearance,[29] compound **63** was synthesized based upon modeling, which suggested a

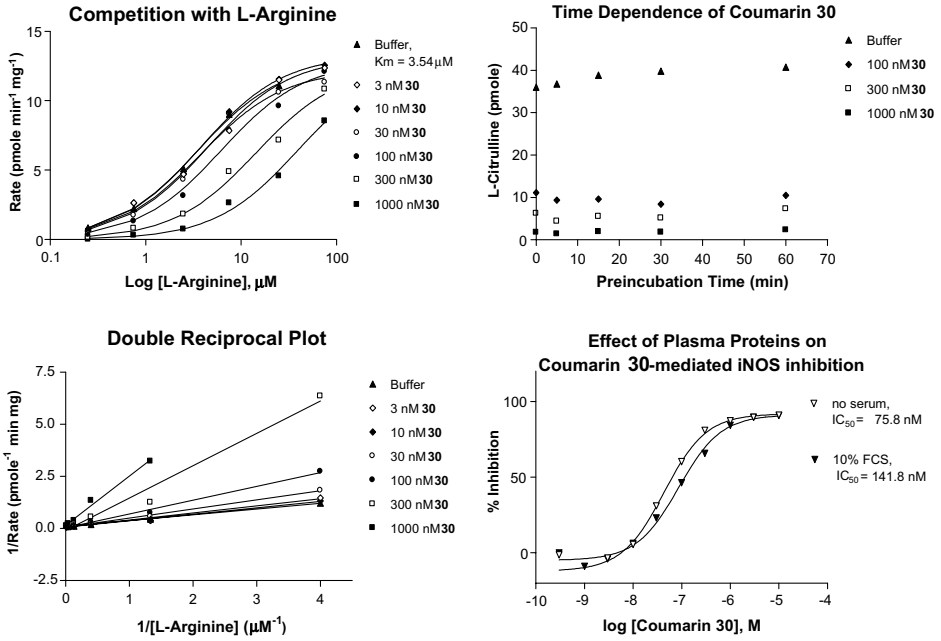
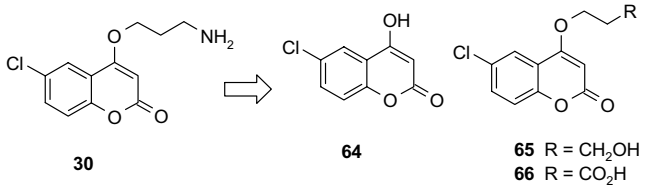


Figure 5. Competition experiments, double reciprocal plot, time dependence and protein binding of coumarin **30**.

Table 4. Pharmacokinetic profile for compounds **30** and **16**

Compound	Species	Dose (mg/kg)	Route	Matrix	AUC 0-inf (ng/h/mL)	*T*1/2 (h)	CL (L/h/kg)	V_{ss} (L/kg)	C_{max} (ng/mL)	T_{max} (h)	*F*
30	Mouse	2	IV	Plasma	153	0.36	13.1	3.58	—	—	—
		2	IV	Brain	209	0.47	—	—	276	0.17	—
		10	PO	Plasma	236	0.77	—	—	368	0.25	0.31
	Rat	2	IV	Plasma	81.5 ± 5.94	0.18 ± .01	25.0 ± 1.75	5.23 ± 0.59	—	—	—
		10	PO	Plasma	161[a]	NC	—	—	111	0.5	0.4
		10	PO	Brain	43.0[a]	NC	—	—	56	0.5	—
16	Mouse	2	IV	Plasma	425	1.75	4.71	10.2	—	—	—
		2	IV	Brain	27.3	0.68	—	—	31.8	0.03	—

[a] AUC 0-7 h; NC = incalculable result.



Scheme 4. Metabolites of **30** from liver microsomes.

small alkyl substituent could be tolerated. It was thought that this substituent might provide steric hindrance to metabolic attack on the ether functionality. However, this compound did not improve on **30**.

4. Conclusion

We found and explored the SAR of a novel series of coumarins as iNOS inhibitors where the usual amidine or guanidine functionality, ubiquitous in the NOS area of study, was absent. This gave rise to coumarin **30** with a respectable human iNOS IC_{50} = 95 nM. These compounds also demonstrated good selectivity over other NOS isoforms. Particularly, coumarin **30** was a competitive inhibitor of human iNOS (K_i = 104 nM) and showed no time-dependency of inhibition. Furthermore, protein binding did not affect the enzyme inhibitory potency. Although we aimed to design a compound that possessed nanomolar cellular potency it is not clear whether the cellular potency of compound **30** is sufficient to demonstrate proof-of-concept in the in vivo models of disease. Given the fact that the PK profile was favorable in terms of bioavailability and brain penetration, further testing of these compounds to demonstrate proof-of-concept in vivo will be undertaken.

5. Experimental

5.1. General

Commercial reagents and solvents were used as received. ^{1}H NMR spectra were recorded on a Varian

MercuryPlus-300 (300 MHz) or Varian Unity Inova (400 MHz) spectrometer as indicated. Proton chemical shifts are reported in δ ppm relative to internal tetramethylsilane (0.0 ppm). MS (LC–MS) data was obtained using a Micromass LCT time of flight mass spectrometer with electrospray ionization and 5 min data acquisition time for *m*/*z* 100–1000. LC (LC–MS) was performed using a Hypersil C18 column (4.6×50 mm, 3 μm with mobile phase of 0.1% TFA in H_2O (A) and 0.1% TFA in ACN (B) and a gradient of 5–100% B over 3 min followed by 2 min at 100% B). Alternatively, a Platform LC–MS with electrospray source may be used with a HP1100 LC system running at 2.0 mL/min, 200 μL/min split to the ESI source with inline HP1100 DAD detection and SEDEX ELS detection. A Luna C18(2) column (30×4.6 mm, 3 μm was used with a gradient of 5–95% B over 4.5 min with mobile phase of 0.1% formic acid in H_20 and 0.1% formic acid in ACN (B). HPLC purification was performed on a Varian ProStar system using a Dynamax reversed-phase C18 column with a linear gradient of ACN/H_2O containing 0.1% TFA. Substituted 4-hydroxycoumarin derivatives were obtained commercially or were synthesized via methods described in the literature.[30] 5-Chloro-4-hydroxycoumarin was synthesized using the procedure of Snieckus and co-workers.[31] Non-commercial Boc-protected alcohols were prepared by reaction of the commercially available amino alcohols with di-*t*-butyldicarboxylate using standard methods.[32] *trans*-2-Methanesulfonyloxymethyl-cyclopropane-carboxylic acid methyl ester was prepared as described previously.[33] Toluene-4-sulfonic acid 6-chloro-2-oxo-2*H*-1-benzopyran-4-yl ester and toluene-4-sulfonic acid 2-oxo-2*H*-1-benzopyran-4-yl ester were prepared by modification of a published procedure.[34] The starting materials for the synthesis of aminoquinolines **61** and **62** was prepared according to the procedure of Kadin and Lamphere.[35] Abbreviations used: dichloromethane (DCM), dimethylformamide (DMF) methanol (MeOH), diethylether (Et_2O), acetonitrile (ACN), ethyl acetate (EtOAc), ethanol (EtOH), trifluoroacetic acid (TFA), tetrahydrofuran (THF), diisopropylazodicarboxylate (DIAD), diisopropylethylamine (DIEA), triphen- ylphosphine (PPh_3), *t*-butyloxycarbonyl (Boc); NMR abbreviations: singlet (s), doublet (d), double doublet (dd), triplet (t), quartet (q), broad (br).

5.1.1. Method A. 6-Chloro-4-(3-aminopropoxy)-1-benzopyran-2-one trifluoroacetic acid salt (30). 6-Chloro-4-hydroxycoumarin (5.0 g, 25.4 mmol), PPh_3 (6.99 g, 26.67 mmol), and (3-hydroxypropyl)-carbamic acid *tert*-butyl ester (4.6 mL, 26.67 mmol) were suspended in 125 mL of THF. The reaction was cooled to −78 °C (smaller scale reactions <5 mmol may be run at 0 °C) and a solution of DIAD (5.48 mL, 27.94 mmol) in 40 mL of THF was added dropwise via an addition funnel at −78 °C. The reaction was allowed to warm slowly to rt and was stirred overnight. The reaction mixture was concentrated in vacuo and Et_2O was added to precipitate a solid, which was filtered, washed with Et_2O, and dried. The solid was treated with neat TFA for 30 min at rt. The TFA was removed under reduced pressure and Et_2O was added to precipitate a white solid, which was filtered, washed with Et_2O, and dried to afford **30** (5.14 g). A second crop (3.1 g) could be obtained by refrigerating the filtrate overnight with an overall combined yield of 88%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.87 (d, 1H, $J = 2.75$ Hz), 7.80 (br s, 2H), 7.73 (dd, 1H, $J = 2.75$, 9 Hz), 7.48 (d, 1H, $J = 9$ Hz), 5.97 (s, 1H), 4.30 (t, 2H, $J = 5.75$ Hz), 3.04 (br s, 2H), 2.12 (m, 2H); MS (ESI, Pos.) calcd for $C_{12}H_{12}ClNO_3$ *m*/*z* [M+H] = 254.1, found 254.1.

5.1.2. Method B. 4-(3-Dimethylamino-propoxy)-1-benzopyran-2-one hydrochloric acid salt (40). 4-Hydroxycoumarin (3.0 mmol, 0.487 g) was dissolved in 10 mL of THF. 3-Dimethylamino-propan-1-ol (0.372 mL, 3.15 mmol) and PPh_3 (0.825 g, 3.15 mmol) were added and the solution was cooled to 0 °C. DIAD (0.647 g, 3.30 mmol) was added dropwise at 0 °C. The reaction was stirred 30 min at 0 °C then allowed to warm to rt and stirred overnight. The solvent was evaporated under reduced pressure, and a solution of anhydrous HCl/MeOH (prepared by adding ~5 mL acetyl chloride dropwise to 100 mL MeOH) was added to the residue. Alternatively 4 M HCl in dioxane may be used. The solvent was removed under reduced pressure and the residue treated with Et_2O. The resulting solid was filtered, washed with Et_2O, and dried to afford **40** (0.485 g, 57%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 10.35 (br s, 1H), 7.90 (dd, 1H, $J = 1.25$, 7.75 Hz), 7.68 (m, 1H), 7.41 (m, 2H), 5.92 (s, 1H), 4.32 (t, 2H, $J = 5.75$ Hz), 3.27 (m, 2H), 2.80 (s, 6H), 2.25 (m, 2H); MS (ESI, Pos.) calcd for $C_{14}H_{17}NO_3$ *m*/*z* [M+H] = 248.1, found 248.1.

5.1.3. Method C. 6-Chloro-4-(3-methylamino-propoxy)-1-benzopyran-2-one trifluoroacetic acid salt (39). 6-Chloro-4-hydroxycoumarin (0.747 mmol, 0.147 g), PPh_3 (0.785 mmol, 0.206 g), and (3-hydroxypropyl)-methyl-carbamic acid *tert*-butyl ester (0.785 mmol, 0.148 g) were suspended in 5 mL THF and the mixture was cooled to 0 °C. DIAD (0.822 mmol, 0.161 mL) was added dropwise at 0 °C, and then the mixture was warmed to rt and stirred for 6 h. The THF was removed under reduced pressure. Neat TFA was added and the solution was mixed at rt for 30 min to 1 h. The TFA was evaporated, Et_2O was added, and the resulting precipitate was filtered. (Alternatively the Et_2O trituration step may be omitted.) The crude material was further purified by HPLC to afford **39** (54.5 mg, 19%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.41 (br s, 2H), 7.87 (d, 1H, $J = 2.5$ Hz), 7.73 (dd, 1H, $J = 2.75$, 9 Hz), 7.48 (d, 1H, $J = 9$ Hz), 5.99 (s, 1H), 4.30 (t, 2H, $J = 5.75$ Hz), 3.14 (m, 2H), 2.63 (t, 3H, $J = 5.5$ Hz), 2.14 (m, 2H); MS (ESI, Pos.) calcd for $C_{13}H_{14}ClNO_3$ *m*/*z* [M+H] = 268.1, found 268.2.

5.1.4. Toluene-4-sulfonic acid 6-chloro-2-oxo-2*H*-1-benzopyran-4-yl ester (14a). 6-Chloro-4-hydroxycoumarin (5.0 g. 25.4 mmol) was suspended in DCM (50 mL) and pyridine (5.1 mL, 63.3 mmol) was added. The mixture was cooled in an ice bath and *p*-toluenesulfonyl chloride (5.1 g, 26.8 mmol) was added. The ice bath was removed and the compound slowly dissolved and a solid is deposited (pyridine·HCl). After 1 h the mixture was transferred to a separatory funnel, HCl (2 M) was

added, and a solid formed. The organic layer was removed and the solid-containing aqueous layer was filtered to remove the precipitate. The filtrate was washed with DCM and the combined organic layers were washed with H_2O, $NaHCO_3$ (satd), H_2O, and brine. The extract was then dried over $MgSO_4$ and was evaporated. The residue was triturated with Et_2O to afford **14a** (8.0 g, 90%) as a cream solid. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.91 (d, 2H, $J = 8$ Hz), 7.54 (d, 1H, $J = 2$ Hz), 7.51 (dd, 1H, $J = 2$, 9 Hz), 7.42 (d, 2H, $J = 8$ Hz), 7.26 (d, 1H, $J = 9$ Hz), 6.38 (s, 1H), 2.49 (s, 3H); LCMS (ACN–H_2O, Luna 3 μm C18(2) 30 × 4.6 mm); MS (ESI, Pos.) calcd for $C_{16}H_{11}ClO_5S$ *m/z* [M+H] = 351 found: [no mass ion detected].

5.1.5. 4-(6-Chloro-2-oxo-2*H*-1-benzopyran-4-yl)-butyronitrile (14b). Toluene-4-sulfonic acid 6-chloro-2-oxo-2*H*-1-benzopyran-4-yl ester (1.05 g, 3 mmol) was dissolved in THF (30 mL) under nitrogen and [P(*o*-tolyl)$_3$]$_2$PdCl$_2$ (0.120 g, 0.15 mmol) was added. 3-Cyanopropylzinc bromide (9 mL of a 0.5 M solution in THF, 4.5 mmol) was added and the resulting yellow solution was stirred at 60 °C for 2 h. Silica gel was added to the dark red reaction mixture, which was concentrated in vacuo and purified by flash column chromatography eluting with CH_2Cl_2 and gradually increasing polarity to 1% MeOH–CH_2Cl_2 to give a residue, which was triturated with Et_2O to afford **14b** (0.54 g, 73%) as a cream solid. ^1H NMR (DMSO-d_6, 400 MHz) δ 7.92 (d, 1H, $J = 2$ Hz), 7.68 (dd, 1H, $J = 9$, 2 Hz), 7.46 (d, 1H, $J = 9$ Hz), 6.47 (s, 1H), 2.90 (t, 2H, $J = 7$ Hz), 2.63 (t, 2H, $J = 7$ Hz), 1.94 (q, 2H, $J = 7$ Hz). LCMS (ACN–H_2O, Luna 3 μm C18(2) 30 × 4.6 mm); (ESI Pos.) calcd for $C_{13}H_{10}ClNO_2$ *m/z* [M+H] = 248; found [no mass ion detected].

5.1.6. 4-(4-Aminobutyl)-6-chloro-1-benzopyran-2-one, acetic acid salt (14). 4-(6-Chloro-2-oxo-2*H*-1-benzopyran-4-yl)-butyronitrile (0.20 g, 0.8 mmol) was suspended in EtOH (20 mL) and a spatula of Raney nickel was added. The mixture was placed under a hydrogen atmosphere and was stirred vigorously at 60 °C for 1 h. The resulting suspension was filtered through Hyflo and concentrated in vacuo. The residue was dissolved in EtOH (5 mL) and treated with HCl (1 M in Et_2O). The resulting solid (128 mg) was purified by flash column chromatography eluting with CH_2Cl_2–MeOH–AcOH–H_2O (240:60:6:4) to afford **14** (85 mg, 27%) as a white solid. ^1H NMR (DMSO-d_6, 400 MHz) δ 7.93 (d, 1H, $J = 2.5$ Hz), 7.68 (dd, 1H, $J = 9$, 2.5 Hz), 7.46 (d, 1H, $J = 9$ Hz), 6.44 (s, 1H), 5.5–4.0 (br s, 3H,), 2.83 (t, 2H, $J = 7$ Hz), 2.68 (m, 2H), 1.78 (s, 3H), 1.65 (m, 2H), 1.54 (m, 2H); LCMS ACN–H_2O, Luna 3 μm C18(2) 30 × 4.6 mm; calcd for $C_{13}H_{14}ClNO_2$ *m/z* [M+H] = 252, found [no mass ion detected].

5.1.7. Toluene-4-sulfonic acid 6-chloro-2-oxo-1,2-dihydro-quinolin-4-yl ester (15a). 6-Chloro-4-hydroxy-1*H*-quinolin-2-one (4.89 g, 25 mmol) was dissolved in DMF (146 mL), tosyl chloride (5.25 g, 27.5 mmol) and triethylamine (8.7 mL, 62.5 mmol) were added and stirred for 1 h to give a precipitate. Water (150 mL) was added and the reaction mixture filtered, the solid was washed with water and then suspended in Et_2O. The solid was filtered and dried in vacuo to afford **15a** (6.0 g, 68%) as a cream solid. ^1H NMR (400 MHz, DMSO-d_6) δ 12.2 (s, 1H), 7.95 (d, 2H, $J = 8$ Hz), 7.62 (dd, 1H, $J = 2.5$, 9 Hz), 7.51 (d, 2H, $J = 8$ Hz), 7.36 (d, 1H, $J = 2.5$ Hz), 7.33 (d, 1H, $J = 9$ Hz), 6.3 (s, 1H), 2.4 (s, 3H); LCMS (ACN–H_2O, Luna 3 μm C18(2) 30 × 4.6 mm); calcd for $C_{16}H_{12}ClNO_4S$ *m/z* [M+H] = 350.0, found (ES+) 349 (M^+), 699 ($2M^+$), 1049 ($3M^+$).

5.1.8. 4-(6-Chloro-2-oxo-1,2-dihydro-quinolin-4-yl)-butyronitrile (15b). Toluene-4-sulfonic acid 6-chloro-2-oxo-1,2-dihydro-quinolin-4-yl ester (1.7 g, 4.84 mmol) and [(*o*-tolyl)$_3$PdCl$_2$ (393 mg, 0.48 mmol) were stirred in THF (100 mL) under nitrogen at room temperature. 3-Cyanopropylzinc bromide (100 mL of a 0.5 M solution in THF, 50 mmol) was added and the reaction mixture was refluxed for 16 h. Silica gel was added to the reaction mixture and concentrated in vacuo. The residue was purified by flash column chromatography eluting with CH_2Cl_2 and gradually increasing polarity to 1% MeOH–CH_2Cl_2. The resulting residue was washed with MeOH and dried in vacuo to afford **15b** (812 mg, 68%) as white solid. ^1H NMR (400 MHz, DMSO-d_6) δ 11.8 (s, 1H), 7.77 (d, 1H, $J = 2.5$ Hz), 7.50 (dd, 1H, $J = 9$, 2.5 Hz), 7.28 (d, 1H, $J = 9$ Hz), 6.39 (s, 1H), 2.83 (t, 2H, $J = 7$ Hz), 2.57 (t, 2H, $J = 7$ Hz), 1.86 (m, 2H); LCMS (ACN–H_2O, Luna 3 μm C18(2) 30 × 4.6 mm) rt = 2.64 min; *m/z* (ES+) 247 (MH^+), 494 ($2MH^+$), 741 ($3MH^+$).

5.1.9. 4-(4-Amino-butyl)-6-chloro-1*H*-quinolin-2-one HCl salt (15). 4-(6-Chloro-2-oxo-1,2-dihydro-quinolin-4-yl)-butyronitrile (0.20 g, 0.81 mmol) was suspended in THF (3 mL) under nitrogen at room temperature. The suspension was treated with borane (2 mL, 1 M in THF), the solid gradually dissolved and the reaction was stirred overnight. The reaction mixture was concentrated in vacuo and the residue dissolved in MeOH (5 mL), then treated with concd HCl in MeOH (1:1) and heated at 70 °C for 1 h. The resulting solid was filtered and dried in vacuo to afford **15** (177 mg, 76%) as a white solid. ^1H NMR (400 MHz, DMSO-d_6) δ 11.8 (s, 1H), 8.0 (br s, 2H), 7.81 (d, 1H, $J = 2.5$ Hz), 7.55 (dd, 1H, $J = 9$, 2.5 Hz), 7.33 (d, 1H, $J = 9$ Hz), 6.45 (s, 1H), 2.85 (t, 2H, $J = 7$ Hz), 2.84 (t, 2H, $J = 7$ Hz), 1.65 (m, 4H); LCMS (ACN–H_2O, Luna 3 μm C18(2) 30 × 4.6 mm); *m/z* (ES+) 251 (MH^+), 502 ($2MH^+$), 753 ($3MH^+$).

5.1.10. 4-(3-Amino-propoxy)-6-chloro-1*H*-quinolin-2-one TFA salt (16). 6-Chloro-4-hydroxy-1*H*-quinolin-2-one (0.513 mmol, 0.100 g), PPh3 (0.539 mmol, 0.141 g), and (3-hydroxypropyl)-carbamic acid *tert*-butyl ester (0.539 mmol, 0.094 g) were dissolved in 5 mL of THF and the solution cooled to 0 °C. Diazodicarboxylate (0.564 mmol, 0.110 mL) was added dropwise at 0 °C and the mixture was stirred at 0 °C for 30 min then warmed to rt. Added an additional 1 equiv of PPh3, DIAD and Boc-protected alcohol and stirred at rt for 2 h. Removed the excess solvent. A portion of the material (1/3) was purified in the Boc-protected form. To the remaining 2/3 of the material, added neat TFA and

stirred at rt for 30 min. The reaction mixture was concentrated in vacuo and Et_2O was added to precipitate a solid, which was further purified by reversed-phase HPLC to afford **16** (29.9 mg, 26%). ^1H NMR (DMSO-d_6) δ 11.52 (s, 1H), 7.80 (d, 1H, $J = 2.25$ Hz), 7.75 (br s, 2H), 7.58 (dd, 1H, $J = 2.25$ Hz, 8.75 Hz), 7.30 (d, 1H, $J = 8.75$ Hz), 5.94 (s,1H), 4.21 (t, 2H, $J = 5.75$ Hz), 3.05 (m, 2H), 2.11 (m, 2H); MS (ESI, Pos.) calcd for $C_{12}H_{13}ClN_2O_2$ *m/z* [M+H] = 253.1, found 253.1.

5.1.11. 4-(3-Dimethylamino-propoxy)-1*H*-quinolin-2-one (17). A suspension of 2,4-dihydroxyquinoline (1.0 mmol, 0.161 g), PPh_3 (1.0 mmol, 0.262 g), 3-dimethylamino-propan-1-ol (1.0 mmol, 0.103 g), and diisopropylazodicarboxylate (1.0 mmol, 0.202 g) in 10 mL of THF was stirred vigorously at rt overnight. The mixture was concentrated under reduced pressure, treated with 1 N HCl (10 mL) followed by extraction with EtOAc (3×50 mL). The aqueous solution was neutralized with 1 N NaOH (15 mL) and extracted with EtOAc (3×100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated to afford **17** as a white solid (0.105 g, 37.2%). ^1H NMR (DMSO-d_6, 300 MHz) δ 11.33 (s, 1H), 7.78 (dd, 1H, $J = 1.3$, 8.25 Hz), 7.51 (m, 1H), 7.27 (d, 1H, $J = 7.75$ Hz), 7.16 (m, 1H), 5.85 (s, 1H), 4.14 (t, 2H, $J = 6.25$ Hz), 2.41 (t, 2H, $J = 7$ Hz), 2.16 (s, 6H), 1.94 (m, 2H); MS (ESI, Pos.) calcd for $C_{14}H_{18}N_2O_2$ *m/z* [M+H] = 247.1, found 247.1.

5.1.12. 4-(3-Amino-propoxy)-1*H*-quinolin-2-one trifluoroacetic acid salt (18). Suspended quinoline-2,4-diol (1.34 mmol, 0.215 g), PPh_3 (1.41 mmol, 0.369 g), and (3-hydroxypropyl)-carbamic acid *tert*-butyl ester (1.41 mmol, 0.24 mL) in 10 mL of THF. Cooled the mixture to 0 °C and then added DIAD dropwise (1.47 mmol, 0.29 mL). Mixed at 0 °C for 10 min then warmed to rt and stirred overnight. Evaporated the solvent under reduced pressure. Added neat TFA and mixed at rt for 30 min. Removed the TFA in vacuo and added Et_2O to precipitate a solid, which was filtered and washed with Et_2O. This material was redissolved in MeOH–H_2O and purified by reversed phase HPLC to afford **18** (0.137 g, 31%). ^1H NMR (DMSO-d_6, 400 MHz) δ 11.40 (s, 1H), 7.86 (br s, 2H), 7.82 (dd, 2H, $J = 1.2$, 8.1 Hz), 7.52 (m, 1H), 7.28 (d, 1H, $J = 7.8$ Hz), 7.17 (m, 1H), 5.88 (s, 1H), 4.20 (t, 2H, $J = 5.9$ Hz), 3.05 (m, 2H), 2.11 (m, 2H). MS (ESI, Pos.) calcd for $C_{12}H_{14}N_2O_2$ *m/z* [M+H] = 219.1, found 219.1.

5.1.13. 4-(3-Amino-propoxy)-1-methyl-1*H*-quinolin-2-one TFA salt (19). Prepared via method C from 4-hydroxy-1-methyl-2(1*H*)-quinolone and (3-hydroxypropyl)-carbamic acid *tert*-butyl ester to afford **19** following HPLC purification (229 mg, 46.3%). ^1H NMR (DMSO-d_6, 300 MHz) δ 7.94 (dd, 1H, $J = 1.5$, 8 Hz), 7.78 (br s, 2H), 7.67 (m, 1H), 7.52 (d, 1H, $J = 8.5$ Hz), 7.28 (t, 1H, $J = 7.25$ Hz), 6.05 (s, 1H), 4.22 (t, 2H, $J = 6$ Hz), 3.57 (s, 3H), 2.04 (m, 2H), 2.12 (m, 2H); MS (ESI, Pos.) calcd for $C_{13}H_{16}N_2O_2$ *m/z* [M+H] = 233.1, found 233.1.

5.1.14. 4-(3-Dimethylamino-propoxy)-1-methyl-1*H*-quinolin-2-one TFA salt (20). Prepared via method C from 4-hydroxy-1-methyl-2(1*H*)-quinolone and 3-dimethylamino-propan-1-ol to afford **20** after HPLC purification (184 mg, 34.4%). ^1H NMR (DMSO-d_6, 300 MHz) δ 9.42 (s, 1H), 7.97 (dd, 1H, $J = 1.5$, 8 Hz), 7.67 (m, 1H), 7.53 (d, 1H, $J = 8.5$ Hz), 7.29 (t, 1H, $J = 7.5$ Hz), 6.06 (s, 1H) 4.21 (t, 2H, $J = 6$ Hz), 3.57 (s, 3H), 3.30 (m, 2H), 2.85 (s, 6H), 2.21 (m, 2H); MS (ESI, Pos.) calcd for $C_{15}H_{20}N_2O_2$ *m/z* [M+H] = 261.2, found 261.1.

5.1.15. 4-(5-Amino-pentyl)-6-chloro-1-benzopyran-2-one (21). 5-(6-Chloro-2-oxo-2*H*-1-benzopyran-4-yl)-pentanenitrile (0.76 mmol, 0.200 g) was suspended in EtOH (20 mL), Raney Nickel (spatula full) was added and the mixture placed under H_2. The mixture was stirred at 60 °C for 1 h. The resulting mixture was filtered through Hyflo and evaporated to leave a yellow gum. The gum was dissolved in EtOH (5 mL) and treated with 1 M HCl in Et_2O (5 mL). No solid deposited. The solvent was evaporated in vacuo and the residue purified by flash column chromatography using CH_2Cl_2–MeOH–AcOH–H_2O (240:30:3:2) as eluent to afford **21** as an off-white solid (80 mg, 39%). ^1H NMR (DMSO-d_6) δ 7.89 (d, 1H, $J = 2.4$ Hz), 7.68 (dd, 1H, $J = 2.4$, 8.8 Hz), 7.46 (d, 1H, $J = 8.8$ Hz), 6.43 (s, 1H), 2.82 (t, 2H, $J = 7.4$ Hz), 2.77 (t, 2H, $J = 7.4$ Hz), 1.67–1.57 (m, 4H), 1.47–1.44 (m, 2H). MS (ESI, Pos.) calcd for $C_{14}H_{16}ClNO_2$ *m/z* [M+H] = 266.1, found 266.05.

5.1.16. 4-(2-Amino-ethoxy)-1-benzopyran-2-one trifluoroacetic acid salt (22). Prepared via method A at 0 °C from 4-hydroxycoumarin and (2-hydroxyethyl)carbamic acid *tert*-butyl ester to afford **22** (250 mg, 59.0%). ^1H NMR (DMSO-d_6, 300 MHz) δ 8.13 (dd, 1H, $J = 1.5$, 8 Hz), 8.09 (br s, 2H), 7.69 (m, 1H), 7.40 (m, 2H), 5.99 (s, 1H), 4.40 (t, 2H, $J = 4.75$ Hz), 3.36 (2H, buried under solvent peak); MS (ESI, Pos.) calcd for $C_{11}H_{11}NO_3$ *m/z* [M+H] = 206.1, found 206.1.

5.1.17. 4-(2-Amino-ethoxy)-6-chloro-1-benzopyran-2-one trifluoroacetic acid salt (23). Prepared via method A at 0 °C from 6-choro-4-hydroxycoumarin and (2-hydroxyethyl)-carbamic acid *tert*-butyl ester to afford 23 (35.8 mg, 9.9%). ^1H NMR (DMSO-d_6, 300 MHz) δ 8.21 (d, 1H, $J = 2.5$ Hz), 8.05 (br s, 2H), 7.72 (dd, 1H $J = 2.5$, 9 Hz), 7.47 (d, 1H, $J = 8.75$ Hz), 6.07 (s, 1H), 4.39 (t, 2H, $J = 4.75$ Hz), 3.33 (2H, buried under solvent peak); MS (ESI, Pos.) calcd for $C_{11}H_{10}ClNO_3$ *m/z* [M+H] = 240.0, found 240.1.

5.1.18. 4-(2-Methylamino-ethoxy)-1-benzopyran-2-one hydrochloric acid salt (24). 4-Hydroxycoumarin (0.216 g, 1.33 mmol) was dissolved in 10 mL of THF. PPh_3 (0.367 g, 1.4 mmol) and (2-hydroxyethyl)-methylcarbamic acid *tert*-butyl ester (0.250 g, 1.44 mmol) was added and the solution was cooled to 0 °C. DIAD (0.287 mL, 1.46 mmol) was added dropwise at 0 °C, and the solution was stirred 15 min. at 0 °C. The reaction was allowed to warm to rt and was stirred overnight. Evaporated the solvent under reduced pressure. Dissolved in neat TFA (10 mL) and mixed at rt for 30 min. The reaction mixture was concentrated in vacuo and was treated with a HCl/MeOH solution followed by Et_2O precipitation to afford **24**, which was filtered, washed with Et_2O, and dried (75 mg, 22.1%). ^1H

NMR (DMSO-d_6, 300 MHz) δ 9.03 (s, 2H), 8.15 (dd, 1H, J = 1.5, 7.75 Hz), 7.69 (m, 1H), 7.40 (m, 2H), 6.00 (s, 1H), 4.49 (t, 2H, J = 4.75 Hz), 3.45 (t, 2H, J = 4.5 Hz), 2.67 (s, 3H); MS (ESI, Pos.) calcd for $C_{12}H_{13}NO_3$ m/z [M+H] = 220.1, found 220.1.

5.1.19. *N*-[3-(6-Chloro-2-oxo-2*H*-1-benzopyran-4-yloxy)-propyl]-methanesulfonamide (25). 4-(3-Aminopropoxy)-6-chloro-1-benzopyran-2-one trifluoroacetate salt (0.272 mmol, 0.10 g) is suspended in 2 mL of DCM. DIEA (1.36 mmol, 0.236 mL) is added followed by methanesulfonyl chloride (0.543 mmol, 0.062 g) and the reaction is stirred at ambient temperature for 4 h. The solvent is removed under reduced pressure and the residue is redissolved and purified by HPLC to afford **25** (21 mg, 23%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.80 (d, 1H, J = 2.6 Hz), 7.70 (dd, 1H, J = 2.5, 8.75 Hz), 7.45 (d, 1H, J = 8.87 Hz), 7.11 (br t, 1H), 5.97 (s, 1H), 4.28 (t, 2H, J = 6 Hz), 3.16 (m, 2H), 2.91 (s, 3H), 2.01 (m, 2H); MS (ESI, Pos.) calcd for $C_{13}H_{14}ClNO_5S$ m/z [M+H] = 332.0, found 331.9.

5.1.20. *N*-[2-(2-Oxo-2*H*-1-benzopyran-4-yloxy)-ethyl]-acetamide (26). 4-(2-Amino-ethoxy)-1-benzopyran-2-one trifluoroacetate **22** (0.157 mmol, 0.050 g) was dissolved in 5 mL of DCM. Pyridine was added (0.314 mmol, 25.4 μL) followed by acetic anhydride (0.314 mmol, 29.6 μL) and the reaction was stirred at rt for 4 h. The reaction mixture was concentrated in vacuo and the residue purified by reversed-phase HPLC to afford **26** (31.4 mg, 81%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.19 (br t, 1H), 7.91 (dd, 1H, J = 1.5, 7.75 Hz), 7.67 (m, 1H), 7.38 (m, 2H), 5.91 (s, 1H), 4.20 (t, 2H, J = 5.5 Hz), 3.52 (m, 2H), 1.84 (s, 3H); MS (ESI, Pos.) calcd for $C_{13}H_{13}NO_4$ m/z [M+H] = 248.1, found 248.0.

5.1.21. *N*-[2-(2-Oxo-2*H*-1-benzopyran-4-yloxy)-ethyl]-guanidine trifluoroacetic acid salt (27). 4-(2-Amino-ethoxy)-1-benzopyran-2-one trifluoroacetate (0.100 g, 0.313 mmol) was placed in a small vial along with 2 mL of acetonitrile. Diisopropylethylamine was added dropwise until the pH of the solution was ~9 by pH paper. *N*,*N*′-Bis(*tert*-butoxycarbonyl)-1-*H*-pyrazole-1-carboxamidine (0.075 g, 0.24 mmol) was added and the reaction was mixed at rt for 5 h. The reaction mixture was concentrated in vacuo, then redissolved in neat TFA and stirred at rt for 30 min. The TFA was removed under reduced pressure and the residue purified by reversed-phase HPLC to afford **27** (35.0 mg, 31%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.88 (dd, 1H, J = 1.5, 8 Hz), 7.81 (br t, 1H), 7.68 (m, 1H), 7.39 (m, 2H), 7.28 (br s, 3H), 5.94 (s, 1H), 4.30 (t, 2H, J = 5 Hz), 3.67 (q, 2H, J = 5.5 Hz); MS (ESI, Pos.) calcd for $C_{12}H_{13}N_3O_3$ m/z [M+H] = 248.1, found 248.1.

5.1.22. 4-(3-Amino-propoxy)-6,7-dimethyl-1-benzopyran-2-one trifluoroacetic acid salt (28). Prepared via method A at 0 °C from 6,7-dimethyl-4-hydroxycoumarin and (3-hydroxypropyl)-carbamic acid *tert*-butyl ester to afford **28** (57.0 mg, 11%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.78 (br s, 2H), 7.59 (s, 1H), 7.23 (s, 1H), 5.81 (s, 1H), 4.28 (t, 2H, J = 6 Hz), 3.04 (m, 2H), 2.33 (s, 3H), 2.29 (s, 3H), 2.11 (m, 2H); MS (ESI, Pos.) calcd for $C_{14}H_{17}NO_3$ m/z [M+H] = 248.1, found 248.2.

5.1.23. 4-(3-Amino-propoxy)-7-methoxy-1-benzopyran-2-one trifluoroacetic acid salt (29). Prepared via method A at 0 °C from 7-methoxy-4-hydroxycoumarin and (3-hydroxypropyl)-carbamic acid *tert*-butyl ester to afford **29** (167.9 mg, 32%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.77 (br s, 2H), 7.75 (d, 1H, J = 8.75 Hz), 7.01 (d, 1H, J = 2.5 Hz), 6.96 (dd, 1H, J = 2.25, 8.75 Hz), 5.75 (s, 1H), 4.28 (t, 2H, J = 6 Hz), 3.86 (s, 3H), 3.02 (m, 2H), 2.10 (m, 2H); MS (ESI, Pos.) calcd for $C_{13}H_{15}NO_4$ m/z [M+H] = 250.1, found 250.1.

5.1.24. 4-(3-Amino-propoxy)-6-bromo-1-benzopyran-2-one trifluoroacetic acid salt (31). Prepared via method C from 6-bromo-4-hydroxycoumarin and (3-hydroxypropyl)-carbamic acid *tert*-butyl ester to afford **31** (64.6 mg, 25%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.98 (1H, d, J = 2.5 Hz). 7.84 (1H, dd, J = 2.5, 9 Hz), 7.76 (2H, br s). 7.40 (1H, d, J = 9 Hz), 5.97 (1H, s), 4.30 (2H, t, J = 5.75 Hz), 3.04 (2H, br s), 2.11 (2H, m); MS (ESI, Pos.) calcd for $C_{12}H_{12}BrNO_3$ m/z [M+H] = 298.0, found [no parent obsd].

5.1.25. 4-(3-Amino-propoxy)-6-fluoro-1-benzopyran-2-one trifluoroacetic acid salt (32). Prepared via method A at 0 °C from 6-fluoro-4-hydroxycoumarin and (3-hydroxypropyl)-carbamic acid *tert*-butyl ester to afford **32** (264 mg, 90%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.79 (br s, 2H), 7.67 (dd, 1H, J = 2.75, 8.75 Hz), 7.52 (m, 2H), 5.97 (s, 1H), 4.30 (t, 2H, J = 5.5 Hz), 3.05 (br s, 2H), 2.10 (m, 2H); MS (ESI, Pos.) calcd for $C_{12}H_{12}FNO_3$ m/z [M+H] = 238.1, found 238.2.

5.1.26. 4-(3-Amino-propoxy)-6-methoxy-1-benzopyran-2-one trifluoroacetic acid salt (33). Prepared via method A at 0 °C from 6-methoxy-4-hydroxycoumarin and (3-hydroxypropyl)-carbamic acid *tert*-butyl ester to afford **33** (195 mg, 65%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.77 (br s, 2H), 7.32 (m, 3H), 5.90 (s, 1H), 4.31 (t, 2H, J = 6 Hz), 3.83 (s, 3H), 3.04 t, (2H, J = 7.25 Hz), 2.12 (m, 2H); MS (ESI, Pos.) calcd for $C_{13}H_{15}NO_4$ m/z [M+H] = 250.1, found 250.2.

5.1.27. 4-(3-Amino-propoxy)-6-methyl-1-benzopyran-2-one trifluoroacetic acid salt (34). Prepared via method A at 0 °C from 6-methyl-4-hydroxycoumarin and (3-hydroxypropyl)-carbamic acid *tert*-butyl ester to afford **34** (298 mg, 94%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.80 (br s, 2H), 7.65 (br d, 1H), 7.49 (dd, 1H, J = 2, 8.5 Hz), 7.31 (d, 1H, J = 8.5 Hz), 5.87 (s, 1H), 4.30 (t, 2H, J = 6 Hz), 3.04 (m, 2H), 2.39 (s, 3H), 2.10 (m, 2H); MS (ESI, Pos.) calcd for $C_{13}H_{15}NO_3$ m/z [M+H] 234.1, found 234.2.

5.1.28. 6,8-Dibromo-4-hydroxy-1-benzopyran-2-one. A solution was prepared of diethylcarbonate (62.5 mmol, 7.6 mL) in 150 mL of toluene to which was added NaH (37.5 mmol, 1.5 g of 60% dispersion in mineral oil) in portions. To this solution was added, dropwise, 3′,5′-dibromo-2′hydroxyacetophenone (12.5 mmol, 3.67 g) in 50 mL of toluene. The reaction was heated to

105 °C overnight, cooled to rt, 100 mL of 1 N NaOH, was added and the reaction was stirred vigorously for 5 h at rt. The layers were separated and the aqueous layer was washed with EtOAc 2×. The combined organic layers were washed with H_2O 1×. The combined aqueous layers were acidified to pH 2 with concd HCl to form a precipitate that was filtered and was washed with H_2O. The solid material was dissolved in ACN/H_2O and speedvacced to dryness to afford **35a** as a light tan solid (0.737 g, 18%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 12.98 (br s, 1H), 8.18 (d, 1H, J = 2.25 Hz), 7.90 (d, 1H, J = 2.25 Hz), 5.64 (s, 1H); MS (ESI, Pos.) calcd for $C_9H_4Br_2O_3$ m/z [M+H] = 318.9, found 318.9.

5.1.29. 4-(3-Amino-propoxy)-6,8-dibromo-1-benzopyran-2-one trifluoroacetic acid salt (35). Prepared via method A at 0 °C from 6,8-dibromo-4-hydroxy-1-benzopyran-2-one and (3-hydroxypropyl)-carbamic acid *tert*-butyl ester. The Boc protected intermediate was HPLC purified prior to treatment with TFA. The title compound **35** was obtained by reversed-phase HPLC purification (38.2 mg, 16.0%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.23 (d, 1H, J = 2.25 Hz), 7.99 (d, 1H, J = 2.25 Hz), 7.75 (br s, 2H), 6.03 (s, 1H), 4.31 (t, 2H, J = 5.75 Hz), 3.04 (m, 2H), 2.11 (m, 2H); MS (ESI, Pos.) calcd for $C_{12}H_{11}Br_2NO_3$ m/z [M+H] = 378.0, found 378.0.

5.1.30. [3-(5-Chloro-2-oxo-2*H*-1-benzopyran-4-yloxy)-propyl]-carbamic acid *tert*-butyl ester. A solution of PPh_3 (0.438 g, 1.67 mmol) in 10 mL of THF was cooled to −78 °C and DIAD (0.430 g) was added in one portion. (3-Hydroxy-propyl)-carbamic acid *tert*-butyl ester (560 mg, 1.3 equiv) in 1 mL of THF was added and the solution was allowed to warm to rt over the course of 1 h. The yellow solution was treated with 5-chloro-4-hydroxycoumarin (0.300 g, 1.5 mmol) and stirred at rt for 3.5 h. The solvent was removed under reduced pressure and the crude product purified using an ISCO 4g SiO_2 column eluting with ether/heptane gradient to afford **36a** (0.106 g, 20%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.27 (m, 3H), 5.71 (s, 1H), 4.73 (br s, 1H), 4.17 (t, 2H, J = 5.75 Hz), 3.41 (q, 2H, J = 6.5 Hz), 2.13 (m, 2H), 1.43 (s, 9H); MS (ESI, Pos.) calcd for $C_{17}H_{20}ClNO_5$ m/z [M+H+Na] = 376.1, found 376.1.

5.1.31. 4-(3-Amino-propoxy)-5-chloro-1-benzopyran-2-one hydrochloric acid salt (36). [3-(5-Chloro-2-oxo-2*H*-1-benzopyran-4-yloxy)-propyl]-carbamic acid *tert*-butyl ester (0.079 g, 0.22 mmol) was treated with 4 N HCl/dioxane for several minutes during which time a precipitate formed. The solvent/HCl was removed under reduced pressure. The residue was recrystallized from MeOH–EtOAc followed by a second recrystallization from EtOH to afford **36** (22.8 mg, 41%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.94 (br s, 3H), 7.62 (t, 1H, J = 8.25 Hz), 7.43 (m, 2H), 5.94 (s, 1H), 4.29 (t, 2H, J = 5.75 Hz), 3.04 (t, 2H, J = 7 Hz), 2.14 (m, 2H); MS (ESI, Pos.) calcd for $C_{12}H_{12}ClNO_3$ m/z [M+H] = 254.0, found 254.1.

5.1.32. 4-(3-Amino-propoxy)-1-benzopyran-2-one hydrochloric acid salt (37). Prepared via method B from 4-hydroxycoumarin and (3-hydroxy-propyl)-carbamic acid *tert*-butyl ester to afford **37** (0.210 g, 50.0%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.03 (br s, 2H), 7.88 (dd, 1H, J = 1.5, 8 Hz), 7.68 (m, 1H), 7.39 (m, 2H), 5.91 (s, 1H), 4.32 (t, 2H, J = 6 Hz), 3.02 (m, 2H), 2.15 (m, 2H); MS (ESI, Pos.) calcd for $C_{12}H_{13}NO_3$ m/z [M+H] = 220.1, found 220.1.

5.1.33. 4-(3-Methylamino-propoxy)-1-benzopyran-2-one trifluoroacetic acid salt (38). Prepared via method A at 0 °C from 4-hydroxycoumarin and (3-hydroxypropyl)-methyl-carbamic acid *tert*-butyl ester to afford **38** (0.380 g, 73.0%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.47 (br s, 2H), 7.89 (dd, 1H, J = 1.5, 8 Hz), 7.68 (m, 1H), 7.40 (m, 2H), 5.91 (s, 1H), 4.31 (t, 2H, J = 5.75 Hz), 3.14 (t, 2H, J = 7.25 Hz), 2.64 (s, 3H), 2.15 (m, 2H); MS (ESI, Pos.) calcd for $C_{13}H_{15}NO_3$ m/z [M+H] = 234.1, found 234.1.

5.1.34. 4-(3-Dimethylamino-propoxy)-7-methoxy-1-benzopyran-2-one hydrochloric acid salt (41). Prepared via method B from 7-methoxy-4-hydroxycoumarin and 3-dimethylamino-propan-1-ol using 4 M HCl in dioxane to form **41** (292 mg, 65.0%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 10.21 (s, 1H), 7.79 (d, 1H, J = 8.75 Hz), 7.01 (d, 1H, J = 2.25 Hz), 6.95 (dd, 1H, J = 2.25, 8.75 Hz), 5.76 (s, 1H), 4.28 (t, 2H, J = 6 Hz), 3.86 (s, 3H), 3.26 (m, 2H), 2.8 (s, 6H), 2.22 (m, 2H); MS (ESI, Pos.) calcd for $C_{15}H_{19}NO_4$ m/z [M+H] = 278.1, found 278.2.

5.1.35. 6-Chloro-4-(3-dimethylamino-propoxy)-1-benzopyran-2-one hydrochloric acid salt (42). Prepared via method B from 6-chloro-4-hydroxycoumarin and 3-dimethylamino-propan-1-ol using 4 M HCl in dioxane to form **42** (332 mg, 73%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 10.23 (s, 1H), 7.88 (d, 1H, J = 2.75 Hz), 7.73 (dd, 1H, J = 2.75, 9 Hz), 7.48 (d, 1H, J = 9 Hz), 6.0 (s, 1H), 4.31 (t, 2H, J = 5.75 Hz), 3.28 (m, 2H), 2.80 (s, 3H), 2.80 (s, 3H), 2.24 (m, 2H); MS (ESI, Pos.) calcd for $C_{14}H_{16}ClNO_3$ m/z [M+H] = 282.1, found 282.1.

5.1.36. 4-(3-Dimethylamino-propoxy)-6-methyl-1-benzopyran-2-one trifluoroacetic acid salt (43). Prepared via method A at 0 °C from 6-methyl-4-hydroxycoumarin and 3-dimethylamino-propan-1-ol. The crude product was purified by HPLC to afford **43** (151 mg, 28.1%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.54 (br s, 1H), 7.64 (br s, 1H), 7.49 (dd, 1H, J = 1.75, 8.5 Hz), 7.31 (d, 1H, J = 8.5 Hz), 5.90 (s, 1H), 4.29 (t, 2H, J = 5.75 Hz), 3.29 (m, 2H), 2.86 (s, 3H), 2.84 (s, 3H), 2.4 (s, 3H), 2.22 (m, 2H); MS (ESI, Pos.) calcd for $C_{15}H_{19}NO_3$ m/z [M+H] = 262.1, found 262.2.

5.1.37. 4-(3-Dimethylamino-propoxy)-6,7-dimethyl-1-benzopyran-2-one trifluoroacetic acid salt (44). Prepared via method A 0 °C from 6,7-dimethy-4-hydroxycoumarin and 3-dimethylamino-propan-1-ol. The crude product was purified by HPLC to afford **44** (63.6 mg, 15.6%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.52 (s, 1H), 7.59 (s, 1H), 7.23 (s, 1H), 5.83 (s, 1H), 4.28 (t, 2H, J = 6 Hz), 3.29 (m, 2H), 2.84 (s, 3H), 2.85 (s, 3H)

2.31 (d, 6H), 2.20 (m, 2H); MS (ESI, Pos.) calcd for $C_{16}H_{21}NO_3$ *m/z* [M+H] = 276.2, found 276.2.

5.1.38. *N*-[3-(2-Oxo-2*H*-1-benzopyran-4-yloxy)-propyl]-guanidine trifluoroacetic acid salt (45). Prepared according to the procedure for *N*-[2-(2-oxo-2*H*-1-benzopyran-4-yloxy)-ethyl]-guanidine from the trifluoroacetic acid salt of 4-(3-amino-propoxy)-1-benzopyran-2-one to afford **45** (40.0 mg, 35%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.88 (dd, 1H, J = 1.5, 8 Hz), 7.67 (m, 2H), 7.39 (m, 2H), 7.15 (br s, 3H), 5.92 (s, 1H), 4.26 (t, 2H, J = 6 Hz), 3.32 (2H, buried under solvent peak), 2.03 (m, 2H); MS (ESI, Pos.) calcd for $C_{13}H_{15}N_3O_3$ *m/z* [M+H] = 262.1, found 262.1.

5.1.39. 1-Methyl-3-[3-(2-oxo-2*H*-1-benzopyran-4-yloxy)-propyl]-thiourea (46). Prepared according to the procedure for 1-[3-(6-chloro-2-oxo-2*H*-1-benzopyran-4-yloxy)-propyl]-3-methyl-thiourea from 4-(3-amino-propoxy)-1-benzopyran-2-one (trifluoroacetic acid salt) to afford **46** (71 mg, 73%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.87 (dd, 1H, J = 1.5, 7.75 Hz), 7.67 (m, 1H), 7.56 (br s, 1H), 7.39 (m, 3H), 5.88 (s, 1H), 4.24 (t, 2H, J = 6 Hz), 3.59 (s, 2H), 2.79 (s, 3H), 2.07 (m, 2H); MS (ESI, Pos.) calcd for $C_{14}H_{16}N_2O_3S$ *m/z* [M+H] = 292.1, found 292.4.

5.1.40. *N*-[3-(6-Chloro-2-oxo-2*H*-1-benzopyran-4-yloxy)-propyl]-guanidine trifluoroacetic acid salt (47). 6-Chloro-4-(3-methylamino-propoxy)-1-benzopyran-2-one trifluoroacetic acid salt (**18**) (0.200 g, 0.543 mmol) was suspended in 7 mL of DCM. Diisopropylethylamine was added dropwise until pH = 9 by pH paper. *N*,*N*′-Bis-(*tert*-butoxycarbonyl)-1-*H*-pyrazole-1-carboxamidine (169 mg, 0.543 mmol) was added and the mixture was stirred at rt overnight. The reaction mixture was concentrated in vacuo and Et_2O was added to form a white precipitate, which was filtered and washed with Et_2O. The crude material was treated with neat TFA (10 mL) for 30 min at rt. The TFA was removed under reduced pressure. Et_2O was added to precipitate the product, which was filtered, washed with Et_2O, and dried. The crude material was further purified by reversed-phase HPLC to afford **47** (108 mg, 48.6%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.86 (d, 1H, J = 2.5 Hz), 7.72 (dd, 1H, J = 2.5, 8.75 Hz), 7.64 (t, 1H, J = 5.5 Hz), 7.47 (d, 1H, J = 8.75 Hz), 7.12 (br s, 3H), 6.00 (s, 1H), 4.26 (t, 2H, J = 5.75 Hz), 3.36 (q, 2H, J = 6.5 Hz), 2.04 (m, 2H); MS (ESI, Pos.) calcd for $C_{13}H_{14}ClN_3O_3$ *m/z* [M+H] = 296.1, found 296.0.

5.1.41. 1-[3-(6-Chloro-2-oxo-2*H*-1-benzopyran-4-yloxy)-propyl]-3-methyl-thiourea (48). 6-Chloro-4-(3-methylamino-propoxy)-1-benzopyran-2-one trifluoroacetic acid salt (**18**) (0.272 mmol, 100 mg) was suspended in 5 mL of 1:1 DMF–DCM. DIEA was added dropwise until pH = 9 by pH paper. Methyl isothiocyanate (0.299 mmol, 0.022 g) was added and the reaction was stirred at rt for 3 h. The reaction mixture was concentrated in vacuo and the crude material was purified by reversed-phase HPLC to afford **48** (66.1 mg, 78.7%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.85 (d, 1H, J = 2.5 Hz), 7.71 (dd, 1H, J = 2.75, 9 Hz), 7.55 (br m, 1H), 7.46 (d, 1H, J = 9 Hz), 5.95 (s, 1H), 4.24 (t, 2H, J = 5.75 Hz), 3.59 (br s, 2H), 2.79 (br s, 3H), 2.07 (m, 2H); MS (ESI, Pos.) calcd for $C_{14}H_{15}ClN_2O_3S$ *m/z* [M+H] = 327.0, found 327.0.

5.1.42. *N*-[3-(6-Chloro-2-oxo-2*H*-1-benzopyran-4-yloxy)-propyl]-acetamide (49). 6-Chloro-4-(3-methylamino-propoxy)-1-benzopyran-2-one trifluoroacetic acid salt (**18**) (0.272 mmol, 100 mg) was suspended in 3 mL of DCM. DIEA (0.544 mmol, 95 μL) was added followed by acetic anhydride (0.816 mmol, 77.1 μL). After stirring for 1 h at rt, the reaction mix was concentrated in vacuo and purified by reversed-phase HPLC to afford **49** (39.0 mg, 49%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.95 (br t, 1H), 7.79 (d, 1H, J = 2.5 Hz), 7.72 (dd, 1H, J = 2.5, 8.75 Hz), 7.46 (d, 1H, J = 8.75 Hz), 5.95 (s, 1H), 4.23 (t, 2H, J = 6 Hz), 3.24 (q, 2H, J = 6 Hz), 1.95 (m, 2H), 1.80 (s, 3H); MS (ESI, Pos.) calcd for $C_{14}H_{14}ClNO_4$ *m/z* [M+H] = 296.1, found 296.2.

5.1.43. 4-(4-Amino-butoxy)-5-chloro-1-benzopyran-2-one hydrochloric acid salt (50). Prepared using the procedure for **36** from [3-(5-chloro-2-oxo-2*H*-1-benzopyran-4-yloxy)-butyl]-carbamic acid *tert*-butyl ester to afford **50** (52 mg, 92%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.86 (br s, 3H), 7.61 (t, 1H, J = 8.25 Hz), 7.42 (t, 2H, J = 9.5, 8.75 Hz), 5.96 (s, 1H), 4.23 (t, 2H, J = 5.75 Hz), 2.85 (m, 2H), 1.83 (m, 4H); MS (ESI, Pos.) calcd for $C_{13}H_{14}ClNO_3$ *m/z* [M+H] = 268.1, found 268.1.

5.1.44. 4-(4-Amino-butoxy)-6-chloro-1-benzopyran-2-one trifluoroacetic acid salt (51). Prepared via method A at 0 °C from and 6-chloro-4-hydroxycoumarin and (4-hydroxy-butyl)-carbamic acid *tert*-butyl ester to afford **51** (185 mg, 59%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.74 (m, 4H), 7.47 (d, 1H, J = 9 Hz), 6.00 (s, 1H), 4.26 (t, 2H, J = 5.75 Hz), 2.89 (m, 2H), 1.87 (m, 2H), 1.74 (m, 2H) MS (ESI, Pos.) calcd for $C_{13}H_{14}ClNO_3$ *m/z* [M+H] = 268.1, found 268.2.

5.1.45. 4-(5-Amino-pentyloxy)-6-chloro-1-benzopyran-2-one trifluoroacetic acid salt (52). Prepared via method A at 0 °C from 6-chloro-4-hydroxycoumarin and (5-hydroxy-pentyl)-carbamic acid *tert*-butyl ester to afford **52** (72.0 mg, 36%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.77 (d, 1H, J = 2.75 Hz), 7.72 (dd, 1H, J = 2.5, 8.75 Hz), 7.65 (br s, 2H), 7.47 (d, 1H, J = 8.75 Hz), 5.99 (s, 1H), 4.23 (t, 2H, J = 6.25 Hz), 2.83 (m, 2H), 1.83 (m, 2H), 1.62 (m, 2H), 1.52 (m, 2H); MS (ESI, Pos.) calcd for $C_{14}H_{16}ClNO_3$ *m/z* [M+H] = 282.1, found 282.1.

5.1.46. 6-Chloro-4-(piperidin-3-ylmethoxy)-1-benzopyran-2-one trifluoroacetic acid salt (53). Prepared via method A at 0 °C from 6-chloro-4-hydroxycoumarin and (3-hydroxymethyl-cyclohexyl)-carbamic acid *tert*-butyl ester to afford **53** (68.1 mg, 23%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.62 (m, 1H), 8.35 (m, 1H), 7.87 (d, 1H, J = 2.75 Hz), 7.73 (dd, 1H, J = 2.5, 8.75 Hz), 7.48 (d, 1H, J = 9 Hz), 6.00 (s, 1H), 4.16 (m, 2H), 3.27 (d, 2H, J = 13 Hz), 2.88 (m, 2H), 2.27 (m, 1H), 1.86 (m, 2H), 1.67 (m, 1H), 1.44 (m, 1H); MS (ESI, Pos.) calcd for $C_{15}H_{16}ClNO_3$ *m/z* [M+H] = 294.1, found 294.2.

5.1.47. 6-Chloro-4-(2-piperidin-2-yl-ethoxy)-1-benzopyran-2-one trifluoroacetic acid salt (54). Prepared via method C at 0 °C from 6-chloro-4-hydroxycoumarin and 2-(2-hydroxy-ethyl)-piperidine-1-carboxylic acid *tert*-butyl ester to afford **54** (43.7 mg, 20.7%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.55 (br s, 1H), 8.36 (br s, 1H), 7.87 (d, 1H, J = 2.5 Hz), 7.73 (dd, 1H, J = 2.5, 8.75 Hz), 7.48 (d, 1H, J = 8.75 Hz), 5.99 (s, 1H), 4.32 (m, 2H), 3.28 (d, 2H, J = 11.75 Hz), 2.91 (m, 1H), 2.10 (m, 4H), 1.76 (br d, 2H), 1.48 (m, 2H); MS (ESI, Pos.) calcd for $C_{16}H_{18}ClNO_3$ *m/z* [M+H] = 308.1, found 308.2.

5.1.48. 6-Bromo-4-(piperidin-4-yloxy)-1-benzopyran-2-one trifluoroacetic acid salt (55). Prepared via method A at 0 °C from 6-bromo-4-hydroxycoumarin and 4-hydroxy-piperidine-1-carboxylic acid *tert*-butyl ester to afford **55** (47.3 mg, 25.3%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.46 (br s, 2H), 8.01 (d, 1H, J = 2.25 Hz), 7.84 (dd, 1H, J = 2.5, 8.75 Hz), 7.41 (d, 1H, J = 9 Hz), 6.14 (s, 1H), 4.97 (m, 1H), 3.32 (2H, buried under solvent peak), 3.14 (m, 2H), 2.12 (m, 2H), 2.01 (m, 2H); MS (ESI, Pos.) calcd for $C_{14}H_{14}BrNO_3$ *m/z* [M+H] = 324.0, found 324.0.

5.1.49. 6-Chloro-4-(pyridin-2-ylmethoxy)-1-benzopyran-2-one trifluoroacetic acid salt (56). Prepared via method B from 6-chloro-4-hydroxycoumarin and pyridin-2-yl-methanol to afford **56** after HPLC purification (36.0 mg, 16.8%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.63 (m, 1H), 7.94 (m, 1H), 7.83 (d, 1H, J = 2.5 Hz), 7.72 (m, 2H), 7.47 (m, 2H), 6.13 (s, 1H), 5.45 (s, 2H); MS (ESI, Pos.) calcd for $C_{15}H_{10}ClNO_3$ *m/z* [M+H] = 288.0, found 287.9.

5.1.50. 6-Chloro-4-(pyridin-3-ylmethoxy)-1-benzopyran-2-one Trifluoracetic acid salt (57). Prepared via method B from 6-chloro-4-hydroxycoumarin and pyridin-3-yl-methanol using HCl/dioxane to form the hydrochloric acid salt, which was then purified by HPLC to afford **57** (45.0 mg, 21.0%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.86 (s, 1H), 8.70 (d, 1H, J = 5 Hz), 8.17 (d, 1H, J = 8 Hz), 7.80 (d, 1H, J = 2.5 Hz), 7.73 (dd, 1H, J = 2.5, 8.75 Hz), 7.66 (m, 1H), 7.49 (d, 1H, J = 9 Hz), 6.17 (s, 1H), 5.46 (s, 2H); MS (ESI, Pos.) calcd for $C_{15}H_{10}ClNO_3$ *m/z* [M+H] = 288.0, found 287.9.

5.1.51. 6-Chloro-4-(pyridin-4-ylmethoxy)-1-benzopyran-2-one trifluoroacetic acid salt (58). Prepared via method B from 6-chloro-4-hydroxycoumarin and pyridin-4-yl-methanol using HCl/dioxane to form the hydrochloric acid salt, which was then purified by HPLC to afford **58** (72.6 mg, 33.0%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.77 (d, 2H, J = 5.75 Hz), 7.94 (d, 1H, J = 2.5 Hz), 7.79 (d, 2H, J = 6 Hz), 7.75 (dd, 1H, J = 2.75, 8.75 Hz), 7.50 d, (1H, J = 9 Hz), 6.10 (s, 1H), 5.56 (s, 2H); MS (ESI, Pos.) calcd for $C_{15}H_{10}ClNO_3$ *m/z* [M+H] = 288.0, found 288.1.

5.1.52. *trans*-2-(6-Chloro-2-oxo-2*H*-1-benzopyran-4-yl-oxymethyl)-cyclopropanecarboxylic acid methyl ester (59). 6-Chloro-4-hydroxycoumarin (1.0 g, 5.09 mmol) was dissolved in 80 mL of DMF, and Cs_2CO_3 (2.49 g, 7.63 mmol), and *trans*-2-methanesulfonyloxymethyl-cyclopropanecarboxylic acid methyl ester (1.28 g, 6.14 mmol) were added. The reaction was heated to 100 °C for 5 h, a further 0.2 equiv of the mesylate was added, and heating was continued for an additional 2 h. After cooling to rt, the DMF was removed in vacuo. The residue was dissolved in EtOAc and washed with H_2O. The organic layer was dried ($MgSO_4$), filtered, and the EtOAc was removed under reduced pressure to afford a yellow solid, which was chromatographed on SiO_2 (35 g) using DCM as the eluent to afford **59** as a white solid (0.98 g, 62%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.75 (m, 2H), 7.45 (d, 1H), 5.97 (s, 1H), 4.22 (m, 2H), 3.65 (s, 3H), 1.90 (m, 2H), 1.19 (m, 2H); MS (ESI, Pos.) calcd for $C_{15}H_{13}ClO_5$ *m/z* [M+H] = 309.0, found 309.0.

5.1.53. *trans*-2-(6-Chloro-2-oxo-2*H*-1-benzopyran-4-yl-oxymethyl)-cyclopropanecarboxylic acid (60). *trans*-2-(6-Chloro-2-oxo-2*H*-1-benzopyran-4-yloxymethyl) cyclpropanecarboxylic acid methyl ester (0.98 g, 3.19 mmol) was dissolved in THF (15 mL). A solution of LiOH (0.134 g, 5.58 mmol) in 5 mL of H_2O was added to the reaction mixture and the mixture was stirred overnight at rt. An additional 0.5 equiv LiOH was added and the mixture was stirred for 1 h. The solution was concentrated and diluted with H_2O, then extracted with Et_2O to remove any unreacted starting material. The aqueous layer was acidified with 3 N HCl and the resulting precipitate was extracted into DCM. The DCM layer was dried ($MgSO_4$), filtered, and evaporated to afford **60** as a white solid (0.367 g, 40%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.25 (br s, 1H), 7.74 (m, 1H), 7.70 (d, 1H, J = 2.5 Hz), 7.46 (d, 1H, J = 8.75 Hz), 6.99 (s, 1H), 4.20 (m, 2H), 1.85 (m, 1H), 1.7 (m, 1H), 1.18 (m, 1H), 1.05 (m, 1H); MS (ESI, Pos.) calcd for $C_{14}H_{11}ClO_5$ *m/z* [M+H] = 295.0, found 295.1.

5.1.54. *N*-(4-Hydroxy-quinolin-2-yl)-acetamide (61a). Prepared as for **62a** to deliver 0.13 g (0.64 mmol, 51%) of a beige solid mp = 297–299 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 10.74 (br s, 1H), 8.017 (d, 1H, J = 4.2 Hz), 7.82 (br s, 1H), 7.682 (d, 1H, J = 4.2 Hz), 7.611 (t, 1H, J = 3.6 Hz), 7.32 (t, 1H J = 3.6 Hz), 5.79 (br s, 1H) 2.15 (s, 3H); MS (ESI, Pos.) calcd for $C_{11}H_{10}N_2O_2$ *m/z* [M+H] = 203.2, found 203.0.

5.1.55. *N*-{4-[3-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-propoxy]-quinolin-2-yl}-acetamide (61b). To a solution of 0.20 g (0.99 mmol) of *N*-(4-Hydroxy-quinolin-2-yl)-acetamide (**61a**) in DMF was added 0.26 g (0.99 mmol) of *N*-(3-bromopropyl)phthalimide followed by 0.39 g (1.2 mmol) of cesium carbonate. The mixture was heated on a steambath under a nitrogen atmosphere for 4 h. Upon cooling a white solid precipitated out, which was collected by filtration. Recrystallization from *i*-PrOH and a minimal amount of methanol delivered 0.29 g (0.74 mmol, 75%) of a white solid mp = 227–9 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 10.67 (s, 1H), 7.80 (m, 5H), 7.67 (d, 1H, J = 4.2 Hz), 7.61 (m, 1H), 7.22 (m, 1H), 4.26 (t, 2H J = 2.7Hz), 3.88 (t, 2H, J = 3.3Hz), 2.50 (m, 2H), 2.12 (s, 3H); MS (ESI, Pos.) calcd for $C_{22}H_{19}N_3O_4$ *m/z* [M+H] = 390.4, found 390.1.

5.1.56. 4-(3-Amino-propoxy)-quinolin-2-ylamine (61). Prepared as for **62** to deliver an unoptimized 0.40 g (0.18 mmol, 48%) of a white solid mp = 175–7 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.81 (dd, 1H, J = 0.6,4.2 Hz), 7.43 (m, 1H), 7.372 (d, 1H, J = 4.2 Hz), 7.09 (m, 1H), 6.21 (s, 2H), 6.19 (s, 1H), 4.168 (t, 2H, J = 3.3 Hz), 2.50 (t, 2H, J = 0.90 Hz), 1.91 (m, 2H). MS (ESI, Pos.) calcd for $C_{12}H_{15}N_3O$ m/z [M+H] = 218.27, found 218.1.

5.1.57. *N*-(6-Chloro-4-hydroxy-quinolin-2-yl)-acetamide (62a). To a solution of 0.85 g (4.34 mmol) of 2-amino-6-chloro-quinolin-4-ol in 30 mL of pyridine is added 1.1 g (10.8 mmol) of acetic anhydride and the mixture heated on a steambath for 2 h under a nitrogen atmosphere. The mixture is concentrated in vacuo and the resulting solid is recrystallized from *i*PrOH to deliver 0.79 g (3.34 mmol, 77%) of a beige solid mp = 365 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 10.66 (br s, 1H), 7.96 (s, 1H), 7.73 (br s, 1H), 7.636 (d, 1H, J = 1.2 Hz), 7.621 (d, 1H, J = 1.2Hz), 5.78 (br s, 1H) 2.14 (s, 3H); MS (ESI, Pos.) calcd for $C_{11}H_9ClN_2O_2$ m/z [M+H] = 236.7, found 237.0.

5.1.58. *N*-{6-Chloro-4-[3-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-propoxy]-quinolin-2-yl}-acetamide (62b). Prepared as for **61b** to deliver an unoptimized 0.10 g (0.23 mmol, 28%) of a beige solid mp = 267–9 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 10.74 (s, 1H), 7.85 (s, 1H), 7.80 (m, 4H), 7.73 (d, 1H, J = 0.9 Hz), 7.67 (d, 1H, J = 4.5 Hz), 7.61 (dd, 1H, J = 1.2, 4.5 Hz), 4.27 (t, 2H, J = 2.7Hz), 3.87 (t, 2H, J = 3.3Hz), 2.50 (m, 2H), 2.13 (s, 3H); MS (ESI, Pos.) calcd for $C_{22}H_{18}ClN_3O_4$ m/z [M+H] = 424.8, found 424.1.

5.1.59. 4-(3-Amino-propoxy)-6-chloro-quinolin-2-ylamine (62). To a solution of 0.16 g (0.38 mmol) of *N*-{6-Chloro-4-[3-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-propoxy]-quinolin-2-yl}-acetamide (**62b**) in ethanol was added 0.4 mL of hydrazine (35% aqueous solution) (4.37 mmol) and the reaction heated on a steambath for 2 h. The reaction mixture was cooled and filtered to remove the phthalhydrazide by-product. The mother liquor was concentrated in vacuo and chromatographed on silica gel (7 N NH_3–MeOH–CH_2Cl_2: 1:9 as eluant) to deliver 0.07 g (0.28 mmol, 74%) of a white solid mp = 167–9 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.74 (d, 1H J = 1.5Hz), 7.43 (dd, 1H, J = 1.2, 4.5 Hz), 7.375 (d, 1H, J = 4.5Hz), 6.38 (s, 2H), 6.24 (s, 1H), 4.17 (t, 2H, J = 3.3 Hz), 2.77 (t, 2H, J = 3.0 Hz), 1.91 (t, 2H, J = 3.3 Hz) MS (ESI, Pos.) calcd for $C_{12}H_{14}ClN_3O$ m/z [M+H] = 252.72, found 252.1.

5.1.60. 6-Chloro-4-hydroxy-3-methyl-1-benzopyran-2-one. Diethylcarbonate (27.2 mmol, 3.3 mL) was dissolved in 30 mL toluene, followed by the addition of NaH (16.3 mmol, 0.65 g of 60% dispersion) in portions. 5′-Chloro-2′hydroxy-propiophenone (5.4 mmol, 1.0 g) was dissolved in toluene (10 mL) and added dropwise via an addition funnel to the above solution. The cloudy yellow-green mixture was heated to near reflux for 20 h then cooled to rt. NaOH (1 N, 50 mL) was added and the mixture stirred at rt for 60 h. The layers were separated and the organic layer washed with H_2O. The combined aqueous layers were washed with Et_2O and the aqueous layer then acidified to pH 2 with concd HCl. The resulting off-white precipitate was filtered, washed with Et_2O, and dried to afford the title compound (0.417 g, 37%). A second crop was obtained by refrigerating the filtrate (0.442 g, 39%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.88 (d, 1H, J = 2.5 Hz), 7.61 (dd, 1H, J = 2.62, 8.87 Hz), 7.40 (d, 1H, J = 8.87 Hz), 2.00 (s, 3H); MS (ESI, Pos.) calcd for $C_{10}H_7ClO_3$ m/z [M+H] = 211.0, found 210.9.

5.1.61. 4-(3-Amino-propoxy)-6-chloro-3-methyl-1-benzopyran-2-one (63). 6-Chloro-4-hydroxy-3-methyl-1-benzopyran-2-one (1.0 mmol, 0.210 g) was suspended in 10 mL THF, and PPh3 (1.05 mmol, 0.275 g) and (3-hydroxy-propyl)-carbamic acid *tert*-butyl ester (1.05 mmol, 0.184 g) were added. The reaction was cooled to 0 °C and DIAD (1.1 mmol, 0.222 g) was added dropwise at 0 °C, and then was warmed to rt and stirred overnight. The THF was removed under reduced pressure to give the Boc-protected intermediate. This intermediate was purified by reversed-phase HPLC and then treated with neat TFA for 5 min. The TFA was removed in vacuo and Et_2O was added to precipitate the product, which was purified by reversed-phase HPLC to afford **63** (116 mg, 30%). ^{1}H NMR (DMSO-d_6, 300 MHz). δ 7.79 (br s, 2H), 7.73 (d, 1H, J = 2.2 Hz), 7.66 (dd, 1H, J = 2.8, 9 Hz), 7.48 (d, 1H, J = 8.8 Hz), 4.29 (t, 2H, J = 6.3 Hz), 3.03 (m, 2H), 2.12 (m, 2H), 2.09 (s, 3H); MS (ESI, Pos.) calcd for $C_{13}H_{14}ClNO_3$ m/z [M+H] = 268.1, found 268.1.

5.2. Enzyme assays

Nitric Oxide Synthase enzymatic assays are based on the [^{3}H]-L-Arginine into [^{3}H]-L-Citrulline conversion originally described by Bredt and Snyder[36] using Km concentration of substrate. Recombinant mouse iNOS, bovine eNOS, and rat nNOS enzymes were purchased from Cayman Chemical (Cat. No. 60862, 60880, 60870). Human iNOS was expressed in *E. coli* at Aventis. The inhibitory activity of each test compound was evaluated by measuring the conversion rate from [^{3}H]-L-Arginine to [^{3}H]-L-Citrulline. Based on this the IC_{50} values were computed for each test compound.

5.3. Enzyme kinetics

Lineweaver–Burk analysis of inhibition of human iNOS by coumarin **30** was performed. Human iNOS activity was measured under initial velocity conditions. Kinetics of coumarin **30** were determined using the radioactive endpoint filtration assay described in the methods. Partially purified human iNOS was incubated in 50 mM Hepes buffer, pH 7.4, with 1 mM NADPH, 15 μM 6*R*-tetrahydrabiopterin, 1 μM FAD, and various concentrations of [^{3}H]-Arginine (0.25, 0.75, 2.5, 7.5, 25, or 75 μM) at 37 °C for 5–10 min. A buffer control, and six concentrations of **30** were used to determine mode of inhibition.

Time-dependency of human iNOS inhibition was examined. Three concentrations of **30** and human iNOS

enzyme were preincubated together for 0, 5, 15, 30, and 60 min prior to initiation of the enzymatic reaction with the addition of NADPH cofactor and arginine substrate. A control with enzyme and no inhibitor was used to show that the enzyme is not losing activity by itself during the preincubation time. Enzyme with 1 mM L-NMA was used as background control. The standard 30 min endpoint assay with [^{3}H]-arginine was used to determine if time-dependent decrease in enzyme activity is observed with coumarin **30**.

5.4. Effect of protein binding on iNOS inhibition

An IC_{50} determination for coumarin **30** with human iNOS was conducted in the presence of 10% fetal calf serum to determine if protein binding has any effect on the activity of the coumarin series.

5.5. Whole cell assays

RAW cells (rodent monocytes) and A172 cells (human glioblastoma) (both available at ATCC) were cultured in DMEM + 10%FBS. Raw cells were plated at 100,000 cells/well in 96-well plates and incubated at 37 °C overnight. The cells were pre-activated by LPS (Sigma) at 1 μg/mL and incubated at 37 °C for 24 h (or overnight). The supernatants were removed. Fresh LPS was added to the cells at 1 μg/mL; meanwhile the inhibitors (nine different concentrations) were added to the cells. The cells were incubated for 24 h. Seventy-five microliters of medium from each well were collected for NO measurement. A172 cells were plated at 30,000 cells/well in 96-well plates and incubated overnight. The cells were pre-activated by LPS (1 μg/mL) + interferon-γ(400 U/mL) + tumor necrosis factor-α (40 ng/mL) + IL-1β (4 ng/mL) and incubated at 37 °C overnight. The supernatant was removed. Fresh LPS + cytokine mix (same concentration as described above) were added to the cells; meanwhile the inhibitors (nine different concentrations) were added to the cells. The cells were incubated for 24 h. Seventy-five microliters of medium from each well were collected for NO measurement. (All cytocykines were ordered from Roche Molecular Biochemicals.)

The Griess reaction was used to measure NO. Sulfanilamide (1 mM, Sigma) and 0.1 N HCl were added to the supernatant and cultured at rt for 10 min. Samples were centrifuged at 1000*g* for 15 min. Seventy-five microliters of the supernatant were transferred into a 96-well Elisa plate. The plates were read absorbance at 548 nm (for background). *N*-(1-Naphthyl)ethylenediamine (1 mM, NEDA, from Sigma) was added to the supernatant and incubated for 10 min at rt. The plates were read absorbance at 548 nm. Pre-NEDA was subtracted from post-NEDA for final reading units.

5.6. Pharmacokinetic studies

Intravenous and oral pharmacokinetics of compounds was characterized in rats and mice. All compounds were dosed as free base equivalents. For rat PK study, ~300 g male, Sprague-Dawley rats were used. The 2 mg/kg ($n = 3$), IV dose was delivered at 1 mL/kg using saline as vehicle. Blood samples were obtained at $T = 0$ (predose), 2, 10, 20, 30 min, and 1, 2, 3, 5, and 7 h post-dose. The 10 mg/kg ($n = 3$ for each time point), PO dose was administered by gavage at 2.5 mL/kg using 0.5% methyl cellulose/0.2% Tween 80 mixture. Blood and brain samples were obtained in a non-serial manner at $T = 0$ (predose), 5, 15, 30 min, and 1, 2, 3, 4, 5 and 7 h post-dose.

For mouse PK studies, ~30 gm female, SJL/J mice were used. Intravenous dose of 2.0 mg/kg and oral dose of 10 mg/kg were administered in similar vehicles as described above. Blood and brain samples were obtained in a non-serial manner from IV dosed mice using similar time schedule. Only plasma samples were obtained from the PO dosed mice.

Sample analysis was performed using LC/MS/MS method. Brains were first homogenized with 3.00 mL of water. A 50.0 μL aliquot of plasma or brain homogenate was transferred to a microcentrifuge tube (1.7 mL), and mixed with 25.0 μL of Internal Standard solution (250 ng/mL of RPR127963 in water) and 1.0 mL of isopropyl ether. The tubes were vortexed at setting 10 for 5 min. and centrifuged at 10,000*g* for 5 min. The organic layer in each tube was transferred into a glass tube (12 × 75 mm) and dried under nitrogen at 37 °C. The residue was reconstituted in 75 μL of 50% acetonitrile in water and transferred into an autosampler vial for LC/MS/MS analysis. LC/MS/MS analysis was performed with a PE Sciex API 3000 mass spectrometer (Analytical column: Inertsil ODS-3, 3 μm, 50 × 2.1 mm with a 10 × 2.1 mm Guard column, Column temperature: ambient, Flow rate: 0.2 mL/min, Injection volume: 15 μL, Mobile phase: 70% of 90% ACN in water + 30% of 0.1% formic acid in water). The lower limit of quantitation was 1 ng/mL for plasma and 2 ng/mL for brain homogenate.

5.7. Metabolism studies

Coumarin **30** was incubated at 25 μM concentration in human and mouse hepatic microsomes for 1 h. Incubations were done both in the presence and absence of NADPH. Identification of potential metabolites was undertaken using LC/MS/MS.

5.8. Docking study

The molecular modeling study was carried out on an SGI workstation running IRIX 6.5 (http://www.sgi.scom). InsightII and Cerius2 from Accelrys (http://www.accelrys.com) was used for most of our molecular modeling studies. ChemX/DiR from Chemical Design LLT (later acquired by Accelrys) was used for binding pose generation and Discover with cff 99 (Accelrys) was used for all molecular mechanics force field calculations and binding pose evaluation.

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