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Synthesis and Evaluation of 4-Hydroxycoumarin Imines as Inhibitors of Class II Myosins

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Author Contributions

C.C., T.W.B., J.S., J.B., A.I., and T.G. wrote or edited the manuscript. J.B. synthesized and characterized all compounds. J.S., K.N., B.M.W., and E.E. performed the ATPase assays and analyzed the data with assistance from R.K.B. C.C. and R.K.B. conducted the motility assays and analyzed the data. D.H. and T.G. did the muscle experiments and analyzed the data. A.I. performed the solubility and stability measurements, recorded all UV/visible spectra in methanol, and determined the λ_{max} and ϵ values.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c01062>.

UV–visible spectra for hydrolysis of **7i** in 99:1 (v/v) PBS/methanol; PDB files for the homology models of bovine cardiac and rabbit skeletal myosins (used for Table 2 and Figure 7) (PDF)

SMILES strings for all of the new compounds tested (CSV)

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Notes

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Abstract

Inhibitors of muscle myosin ATPases are needed to treat conditions that could be improved by promoting muscle relaxation. The lead compound for this study ((3-(*N*-butylethanimidoyl)ethyl)-4-hydroxy-2*H*-chromen-2-one; BHC) was previously discovered to inhibit skeletal myosin II. BHC and 34 analogues were synthesized to explore structure–activity relationships. The properties of analogues, including solubility, stability, and toxicity, suggest that the BHC scaffold may be useful for developing therapeutics. Inhibition of actin-activated ATPase activity of fast skeletal and cardiac muscle myosin II, inhibition of skeletal muscle contractility *ex vivo*, and slowing of *in vitro* actin-sliding velocity were measured. Several analogues with aromatic side arms showed improved potency (half-maximal inhibitory concentration (IC₅₀) <1 μ M) and selectivity (12-fold) for skeletal myosin *versus* cardiac myosin compared to BHC. Several analogues blocked neurotransmission, suggesting that they are selective for nonmuscle myosin II over skeletal myosin. Competition and molecular docking studies suggest that BHC and blebbistatin bind to the same site on myosin.

Graphical Abstract

INTRODUCTION

Myosins are essential actin-activated motor proteins that hydrolyze adenosine triphosphate (ATP) to produce motion and force under load.¹ Class II myosins are found in the contractile apparatus and are responsible for voluntary and involuntary muscle contraction. Distinct isoforms are found in skeletal, cardiac, and smooth muscles.² Differences in kinetics of the ATPase cycle (Figure 1) tune physiological parameters specific to muscle function such as contractile speed, maximal force, and force maintenance.³ There are also nonmuscle myosin II isoforms that are found in stress fibers of nonmuscle cells. They mediate diverse cellular processes such as cell division, migration, and adhesion. Class II myosins share a conserved motor domain containing the actin-binding and ATP-binding sites, a lever arm domain to amplify motion, and a tail domain to allow filaments to form through intermolecular interactions (Figure 1).⁴

Under activating cellular conditions, myosin cycles between actin-attached and actin-detached states, controlled by the interaction of ATP with myosin, which weakens actin-binding affinity (Figure 1). After conformational changes triggered by ATP hydrolysis, myosin weakly binds to actin and upon release of inorganic phosphate (P_i) transitions to a strongly bound adenosine diphosphate (ADP) state, which undergoes the working step, resulting in relative motion between actin and myosin or in the generation of force.³

Several clinical conditions are characterized by excessive muscle tone or activity. The examples include spasticity of skeletal muscles as a result of genetic mutations⁵ or spinal cord injuries⁶ and cardiomyopathies caused by mutations in cardiac sarcomeric proteins including myosin.⁷ Several conditions are caused by excessive bronchial, gastrointestinal, and urinary smooth muscle contractility, such as asthma,⁸ diarrhea, and overactive bladder.⁹ In some of these conditions, genetic alterations directly lead to dysregulated myosin activity,¹⁰ whereas in others, excess myosin activity is an indirect consequence of neural, immune, or other dysfunction.

As a result of this central role in mediating muscle contractility, myosins have emerged as an appealing target for small-molecule inhibitors aimed at ameliorating muscle hypercontractility.^{11,12} However, myosin II is a difficult target due to its presence in three different kinds of muscle as well as nonmuscle cells. To promote muscle relaxation, inhibitors must stabilize myosin states that interact weakly with actin (Figure 1). Kinetically, this means that the rate-limiting step in the ATPase cycle, k_{att} , must be slowed, trapping myosin in the ADP- P_i state. One of the best-characterized inhibitors, blebbistatin (**1a**, Figure 2, review¹³), inhibits k_{att} for skeletal, cardiac, and vertebrate nonmuscle myosins in the low micromolar range, but is less potent for smooth muscle myosin. Photostability, phototoxicity, and low solubility of blebbistatin¹¹ have led to searches for analogues with more favorable physicochemical properties,^{14–19} such as the less potent *para*-aminoblebbistatin (**1b**) with improved photostability, solubility, and phototoxicity.^{14,15,19} The most selective myosin inhibitor known (CN-2018571)²⁰ targets smooth muscle myosin II and binds to a different site than blebbistatin.

The objective of this work was to synthesize and characterize novel, small-molecule myosin inhibitors as potential therapeutics for patients with symptoms of diseases or syndromes that could be improved by promoting muscle relaxation. The results of a small-molecule library screen²¹ showed that the compound 3-(*N*-butylethanimidoyl)-4-hydroxy-2*H*-chromen-2-one (BHC, **2**, Figure 2) exhibited a detectable but unspecified decrease in the actin-activated ATPase activity of skeletal muscle myosin at 100 μ M. However, no further properties were investigated, including the half-maximal inhibitory concentration (IC₅₀), mechanism of action, or selectivity toward different muscle myosin isoforms. Subsequently, we demonstrated that **2** selectively blocks nerve-evoked contraction of mouse skeletal muscle without affecting neurotransmitter release,²² which is required for the action potential. This established a new tool to directly evaluate neurotransmission by measuring successfully transduced muscle action potentials without interference by muscle movement, which damages the electrodes and prevents accurate readings. These useful properties of **2** were not found for the myosin inhibitor *N*-benzyl-*p*-toluenesulfonamide (BTS),²¹ which blocked neurotransmission in addition to muscle movement.²²

Structural similarities between blebbistatin (**1a**) and BHC (**2**) shown in Figure 2 suggest that they share the same pharmacophore and bind to the same site of myosin II. In this study, we synthesized and characterized 2 and 34 analogues, then examined them for their ability to inhibit myosin function using two *in vitro* assays and one *ex vivo* muscle contractility assay. We have determined the IC₅₀ values for inhibition of actin-activated ATPase activity for both fast skeletal and cardiac muscle myosin. From *in silico* molecular modeling and by competition assays, we provide evidence that **2** and an analogue of **2** bind to the same site on myosin as blebbistatin (**1a**). Interestingly, several analogues exhibit higher affinity and are more selective for skeletal or cardiac muscle myosin than either **2** or **1a**, which are of comparable potency. As predicted by our prior kinetic models,^{23–25} **2** slows *in vitro* actin-sliding velocity with IC₅₀ values that are similar to those for inhibition of actin-activated ATPase, suggesting a common kinetic mechanism of action for both effects. Finally, by combining information from *in vitro* and *ex vivo* assays, several analogues appear more selective for nonmuscle myosin than either skeletal or cardiac myosin.

RESULTS AND DISCUSSION

Synthesis.

Previous studies on blebbistatin analogues have found that variation of the hydrophobic side chain region can tune relative inhibitory potency for myosins. According to the proposed pharmacophore comparison shown in Figure 2, this corresponds to the butylimine side chain of BHC (**2**), so this became the initial focus of analogue studies. Preparation of the Schiff base BHC (**2**, Figure 2) by condensation of butylamine with 3-acetyl-4-hydroxycoumarin (3-acetyl-4-hydroxy-2*H*-chromen-2-one, **4**, Scheme 1) was previously reported.²⁶ Bicyclic compound **4**²⁷ is a scaffold for several biologically active compounds and is readily prepared from inexpensive 4-hydroxycoumarin (4-hydroxy-2*H*-chromen-2-one, **3**) by base-catalyzed acetylation with acetyl chloride²⁸ or by reaction with acetic acid or acetic anhydride and phosphorus oxychloride.²⁹ As shown in Scheme 1, the latter method was used to prepare **4**, as well as the 3-propionyl (**5a**) and 3-butanoyl (**5b**) analogues. Given the ease of condensing

these ketones with various commercially available amines³⁰ or their hydrochloride salts,³¹ a series of 20 BHC analogues (**6a–k** and **7a–i**) were prepared from **4** with various imine side chains to explore structure–activity relationship (SAR). The *N*-benzyl analogue **7a** was found early in our studies to have promising inhibitory activity, so we also prepared analogues **8a** and **8b** replacing the acetyl methyl group with bulkier ethyl and propyl groups, respectively. Most of these compounds were isolated in high yields after recrystallization to obtain high purity. Another hit from the same small-molecule screen as BHC (**2**),²¹ 5-nitrocoumarin **10a** bearing a pentyl side chain, was synthesized as shown in Scheme 2 *via* nitration³² of 3-acetyl-4-hydroxy-2*H*-chromen-2-one (**4**), followed by condensation with pentylamine. Since the *N*-benzyl analogue **7a** lacking a nitro substituent showed activity in initial studies, intermediate **9** was also condensed with benzylamine to give compound **10b**. Nitro groups are often associated with toxicity issues in drugs,³³ so further nitro analogues were not pursued.

Quinoline and quinolinone ring systems are favorable scaffolds in drug candidates,³⁴ so a series of 4-hydroxyquinolin-2-one analogues were synthesized (Scheme 3). Replacing the 1-position oxygen atom of BHC with nitrogen should increase the dipole moment of the ring system and also increase the hydrogen bond acceptor (HBA) ability of the 2-position carbonyl oxygen. Key intermediate, 3-acetyl-4-hydroxyquinolin-2(1*H*)-one (**11**), was prepared from inexpensive starting materials ethyl acetoacetate and methyl anthranilate.³⁵ The low potency of **12a–i** (*vide infra*) suggested that replacing the oxygen in the coumarin series (**2**, **6**, and **7**) with an NH group may perturb binding by introducing a hydrogen bond donor (HBD) in the 1-position of the ring system. Accordingly, compound **14**, an *N*-methyl analogue of **2** and **12c**, was prepared, as shown in Scheme 3.

Structure.

Schiff bases derived from 3-acyl-4-hydroxycoumarins or 3-acyl-4-hydroxyquinolin-2-ones can exist in either of two tautomeric forms, labeled “imine” and “enamine” in Figure 3. While both can be stabilized by intramolecular hydrogen bonding, X-ray crystal structures of **6j** and **7a** show only the enamine isomers,^{36–38} and the NH–O hydrogen bond in the enamine form of **7a** has been studied computationally.^{36,38,39} In addition, the structures of **6a**, **6j**, and **7a** were assigned as enamine tautomers from their ¹H and ¹³C NMR spectra in CDCl₃ and dimethyl sulfoxide (DMSO)-*d*₆.⁴⁰

O-Benzyloxime **7e** and phenylhydrazine **7f** should exist primarily as imine tautomers under most conditions, but the prevalent isomer of **2**, **6a–k**, **7a–d**, **7g–i**, **10a**, **10b**, **8a**, **8b**, **12a–i**, and **14** in solution could depend on solvent characteristics. In CDCl₃ solution, a single peak at $\delta \sim 181$ ppm for the ketone carbonyl carbon is observed in the ¹³C NMR spectra for the enamine structures of **6a**, **6c**, **6d**, and **6g**. As in the crystal structures, intramolecular hydrogen bonding should stabilize the enamine tautomers in chloroform. DMSO is a strong HBA and could break the intramolecular hydrogen bond, but in DMSO-*d*₆ compounds **2**, **6a–k**, **7a–d**, **7g–i**, **10a**, **10b**, **8a**, **8b**, **12a–i**, and **14** consistently show a ketone carbonyl resonance at $\delta \sim 180$ ppm in the ¹³C NMR spectra and HNCH splitting is observed in the ¹H NMR spectra. To maintain consistency with the nomenclature in the previous literature, these compounds are named as imine tautomers in the Experimental Section, but the

assignments for the ^1H NMR spectra are based on the enamine structures. Consistent with the expectation that *O*-benzyloxime **7e** and phenylhydrazine **7f** would exist primarily as the imine tautomer, their NMR spectra showed no ketone carbonyl resonance and no HNCH splitting. These observations are relevant to the computational docking studies because the locations of the HBD and HBA groups on the small molecules affect specific interactions with amino acids forming the binding site.

Structure–Activity Relationships.

To evaluate the relative ability of the compounds to inhibit myosin II function, we measured the IC_{50} values for reduction of steady-state actin-activated Mg^{2+} ATPase activity for skeletal and cardiac myosin. This assay measures the rate-limiting step in the acto–myosin ATPase cycle (k_{att} , Figure 1), which is the kinetic parameter most related to the ability to inhibit muscle contraction. We chose to use an assay that directly measures production of P_i with time, detecting absorbance in a stoppedtime format. This avoids potential interference by light scattering or fluorescence. Figure 4A shows the representative plots of ATPase activity *versus* concentration of selected BHC analogues.

Table 1 contains all IC_{50} data obtained from a fit to a four-parameter logistics equation as well as the number of replicates (N), *i.e.*, the number of times a fully independent titration/ATPase assay was performed. The IC_{50} value for **1a** was within the range of published values for skeletal myosin (0.01–4 μM) and cardiac myosin (0.4–30 μM).¹⁵ Values for **1b** were similarly within the range.¹⁴ Both compounds were more potent inhibitors of skeletal *versus* cardiac myosin by about a factor of 4.5.

Table 1B shows data for compounds with linear aliphatic groups appended to the imine nitrogen. BHC (**2**) was found to be a less potent inhibitor than **1a** and **1b** by about a factor of 3. However, it fits the same trend as the blebbistatins, being ~ 3 -fold more potent for skeletal *versus* cardiac myosin. Increasing the aliphatic chain length did not increase this selectivity but had a significant effect on the potency for both skeletal and cardiac myosin (Figure 4B). Compounds having side arms with fewer than four carbons were poor inhibitors. Increasing the chain length from four to six carbons gave at most (**6i**) ~ 2 -fold increase in potency over **2**. Appending a terminal OH group had little effect on the potency of selectivity. Branching at the α - or β -carbon of the side arm did not improve the inhibitory potency of the BHC analogues with less than four carbons (Table 1C).

Benzyl (**7a**) and 4-methylbenzyl (**7d**) analogues of **2** showed a ~ 10 -fold higher potency for skeletal than for cardiac myosin and a ~ 2 -fold improvement in potency over **2** for skeletal myosin (Table 1D). Both of these effects were absent in the 3-methylbenzyl analogue **7c** and the 2-methylbenzyl analogue **7b**, which lacked significant potency. Because of this sensitivity to modification, further derivatives of **7a** were prepared to examine the effect of lengthening the imine side arm, but this caused a loss of potency as in **8a** and **8b**. Derivatives with insertion of a heteroatom (**7e** and **7f**) did not lead to improvements in potency relative to **7a**, although **7f** retained high selectivity for skeletal myosin. Notably, isobutylimine **7c** and *m*-methylbenzylamine **7c** displayed some degree of selectivity for cardiac *versus* skeletal myosin, suggesting the potential to develop drugs for cardiomyopathies.

The pyridine analogues, **7g–i** (Table 1E), all showed a similar trend toward decreased potency relative to **7a** but largely for cardiac myosin. The 4-pyridylmethyl analogue **7i** and phenylhydrazine **7f** showed the highest selectivity of any derivatives in Table 1 with 12-fold preference for skeletal *versus* cardiac myosin.

None of the quinolinone derivatives, **12a–i**, **14** (Table 1F), had measurable potency toward cardiac or skeletal myosin, showing that even with favorable imine side arms, the oxygen in the 1-position is critical to inhibiting ATPase activity and cannot be replaced with a NH or *N*-methyl as in **14**. Similarly, the BHC analogues with a 6-nitro group (Table 1G) **10a** and **10b** lacked potency; **10b** was previously found to have detectable but unspecified inhibitory activity for skeletal myosin (at 100 μM) in the small-molecule screen that uncovered BHC,²¹ but we find that it is a poor inhibitor for both skeletal and cardiac myosins (IC_{50} 100 μM), in agreement with our previous demonstration of its inability to paralyze the skeletal muscle tissue.^{4,22}

Effects of BHC on Velocity of Actin Sliding in an *In vitro* Motility Assay.

Another measure of myosin function can be assessed using the *in vitro* motility or actin-sliding assay in which fluorescent actin filaments are observed moving over a bed of myosin attached to a coverslip.⁴¹

Figure 5 shows that BHC inhibits actin-sliding velocities with an IC_{50} of $2.8 \pm 0.4 \mu\text{M}$, a value very close to the IC_{50} for the ATPase data ($2.1 \pm 0.8 \mu\text{M}$). This finding is predicted by our work showing that velocity is not solely detachment limited in this assay but rather also depends on the acto–myosin attachment rate, which is limited by the ATPase activity.^{23–25}

Mechanism of Interaction with Myosin.

Since blebbistatin (**1a**) and potent BHC analogues inhibit ATPase activity and *in vitro* motility, we determined whether they bind to the same site on myosin. In the search for a simple method to measure binding, we discovered that **1a** shows a ~40-fold increase in fluorescence emission at 560 nm (λ_{ex} 440 nm) upon saturation with the soluble head domain (S1) of skeletal myosin, but only in the presence of 1 mM ATP (Figure 6A).

This is consistent with the kinetic studies, showing that **1a** has a high affinity only for the myosin–ADP– P_i transition state.⁴² Without ATP, **1a** fluorescence remained at background levels (data not shown). S1 was added to a fixed concentration of **1a** and data were fit to the quadratic form of the equation for a single-site binding equilibrium to give K_d for **1a** = $0.4 \pm 0.1 \mu\text{M}$ (Figure 6A). This value is essentially the same as the IC_{50} of **1a** for skeletal myosin (Table 1).

Compound **7a** was selected as a representative compound to determine whether the BHC analogues can compete with **1a** for binding to myosin. In contrast to **1a**, the weak fluorescence of **7a** (λ_{ex} 320 nm, λ_{em} 400 nm) did not change upon adding S1 (with MgATP; data not shown). Figure 6B shows that adding increasing **7a** to a mixture of S1 and **1a** caused the fluorescence of **1a** to decrease, suggesting that **7a** and **1a** compete for the same binding site on S1. A fit to a four-parameter logistics equation gives an estimate of the IC_{50} value of **7a** for S1 of $5.3 \pm 1.1 \mu\text{M}$ with a slope = 0.8. The K_i (apparent K_d) was calculated

from the IC₅₀ value using the equations described⁴³ or using the IC₅₀-to-*K_i* converter (https://bioinfo-abcc.ncifcrf.gov/IC50_Ki_Converter). Free **7a** concentrations were applied to a competitive binding model, giving *K_i* = 0.8 ± 0.2 μM, which is essentially the same value as the IC₅₀ for inhibition of the ATPase activity (Table 1D). These data suggest that **1a** and **7a** compete for the same binding site on S1 with similar affinity.

Computational Modeling.

The data shown in Figure 6 suggest that **7a** and blebbistatin (**1a**) bind to the same site in skeletal myosin. The atomic resolution structure of the motor domain of *Dictyostelium discoideum* myosin (PDB 1yv3⁴⁴) shows that **1a** binds to a hydrophobic pocket at the apex of the open conformation of the cleft between the upper 50 and lower 50 kDa subdomains of the motor domain (Figure 7C). The cleft must close to allow strong binding to actin,⁴⁵ explaining why **1a** inhibits the release of P_i from the ADP-P_i complex, which is normally accelerated by actin binding (Figure 1).

To evaluate the structural aspects of analogue binding, we prepared two homology models (cardiac and skeletal myosin) using 1yv3 as the template and compared *in situ* docking to the models and template. The highest scoring pose from docking **1a** to 1yv3 was essentially the same as the known structure (data not shown).

Docking to the cardiac structure showed that **1a** (Figure 7A) and **2** (Figure 7B) bound to the same site as **1a** in 1yv3. Table 2 summarizes the contact residues and interactions found from docking **1a** and **2**, which share a majority of residue interactions. Ala463, located directly adjacent to ATP on Switch 2 next to P_i in the ADP-P_i transition state, as well as Gly245, and Leu267 form hydrogen bonding interactions with **1a** (Figure 7A). Two of these residues also participate in polar interactions with **2**, specifically the amide nitrogen of Ala463 and amide oxygen of Leu267 (Figure 7B). Molecule **1a** forms π-stacking interactions between the A-ring and Tyr266 with parallel aromatic planes at 4.2 Å, as well as an edge-to-face π interaction between the D-ring and Phe473, with perpendicular aromatic rings at 4.9 Å. These distances are within the range of known π-π interactions.⁴⁶ Comparable π-π interactions are also formed between the aromatic coumarin core of **2** and the phenyl ring of Tyr266, at the same distance as the interaction between Tyr266 and **1a** (Figure 7A).

Docking with both the imine and enamine tautomers of **2** (Figure 3) suggests that the enamine has the most favorable interactions. The highest scoring pose for the enamine shows the carbonyl oxygen at the 4-position near a HBD group, the protonated imidazole ring of His651. The carbonyl oxygen in the enamine is a more favorable HBA than the hydroxyl group at this position in the imine. The nitrogen of **2** was also near an HBA, the amide oxygen of Leu267 (Figure 7B). Also, the planar conformation of the enamine better fits the shape of the pocket. The enamine resulted in higher scoring poses than the imine, which is consistent with the prevalence of the enamine tautomers for the BHC compounds, as shown by the NMR spectra as previously discussed.

The region of the pocket surrounding the variable imine side arm of the BHC analogues is hydrophobic, consisting of aliphatic regions of Val647, Phe473, Glu474, Lys270, Glu269,

and Leu267 in cardiac myosin (Figure 7D). Similar to the *N*-phenyl group of **1a**, the face of the phenyl group of **7a** is at a near-right angle to the aromatic plane of Phe473. This capacity for edge-to-face aromatic interactions could explain differential affinity for certain BHC analogues having aromatic rings in their side arms, but does not explain selectivity for skeletal *versus* cardiac myosin (*e.g.*, **7a**). Longer aliphatic or aromatic rings are predicted to have more potential for hydrophobic interactions, generally consistent with correlation between increasing aliphatic side chain length and greater potency for ATPase inhibition (Figure 4B). These residues surrounding the side arm are mostly conserved, except a leucine residue in skeletal myosin is at the position of Phe473 in cardiac myosin (Table 2). The only other residue difference between the binding pockets is the replacement of the His651 residue in cardiac by phenylalanine in skeletal myosin.

Docking experiments using the two homology models showed that **1a** and most of the analogues (enamine and imine tautomers) docked to the 1a binding site in the highest scoring pose. Interesting exceptions were **6h**, **6i**, **10a**, **10b**, **8a**, and **8b**, which each docked to unique locations. Inspections of the structures (Table 1) show that they contain either long side arms (**6h** (5 C), **6i** (6 C)), nitro groups on the coumarin ring (**10a**, **10b** (5 C)), or an extended side arm attached to the imine carbon (**8a** (2 C), **8b** (3 C)). Of these analogues, only **6h** and **6i** have good potency by ATPase assay. Lack of docking to the **1a** binding site may suggest that the site must rearrange to accommodate the long side arm.

Physiological Studies.

Our biochemical and modeling results suggest that BHC analogues and **1a** bind to the same site (Figures 6 and 7). ATPase data show that some compounds are selective for skeletal *versus* cardiac myosin (like **1a**), have opposite selectivity (*e.g.*, **6f** and **7c**, Table 1), or lack selectivity. To test whether the analogues exhibit additional selectivity for the predominant class II myosins found in nerves, nonmuscle myosin IIB, as well as to examine their effects in *ex vivo* muscle contractility assays, we took advantage of the mouse phrenic nerve–hemidiaphragm preparation. The hemidiaphragm can be stimulated indirectly through electrical pulses delivered through a suction electrode attached to the phrenic nerve. This allows for simultaneous evaluation of the effects of the compounds on skeletal muscle contractility and on neurotransmission, which depends on nonmuscle myosin IIB.⁴⁷

We measured contractility optically as the diaphragm muscle fiber shortening in response to stimulation of the phrenic nerve²² in the presence of the selected compounds (100–300 μ M). Figure 8 shows a representative example of the contractility data of the muscle treated with **7i**, which essentially blocked muscle contraction at 100 μ M. Next, in the case where analogues produced >85% block in contractility at any dose, we attempted to record changes of transmembrane membrane potential in muscle in response to phrenic nerve stimulation in the presence or absence of the compounds. We reasoned that if a drug selectively blocked skeletal muscle myosin but not nonmuscle myosin IIB, we should be able to record muscle action potentials, since these electrical potentials depend on the activity of voltage-gated sodium channels upstream of calcium release and subsequent actin–myosin interactions.

Compound **7i** effectively paralyzed muscle at 100 μM without blocking neurotransmission (Table 3), as we have previously shown for **2**.²² Thus, **2** and **7i** form class 1 in Table 3, which inhibit skeletal muscles but not nerve nonmuscle myosin IIB. This important selectivity has not been demonstrated to date for other known myosin inhibitors to our knowledge. Compared to **2**, **7i** has a much higher potency for skeletal than cardiac myosin as well, an attractive property for treating skeletal hypercontractility.

Class 2, which includes **1a**, effectively paralyzed the diaphragm muscle at 100 μM , generally consistent with the potency assessed by ATPase activity. However, action potentials were also blocked (Table 3), consistent with the inhibitory effects of **1a** on nonmuscle IIB⁴⁸ as well as on synaptic transmission previously reported.⁴⁷ It is likely that these compounds have potency for both skeletal myosin and nonmuscle myosins in the nerve terminal.

Class 3 analogues are similar to class 2 with respect to observed muscle effects, but have poor potency with respect to ATPase activity. This reduction in contractility is therefore likely a consequence of the upstream effect to block nerve activity, suggesting that class 3 analogues are likely selective for nonmuscle myosin IIB over skeletal myosin. Interestingly, all compounds in this class are also poor inhibitors of cardiac myosin (Table 1, IC_{50} 100) and therefore appear to be relatively selective for nonmuscle IIB. Among these are five quinolinones (**12d**, **12e**, **12f–h**), but not all quinolinones tested have this property (**12a–c**, **12i**). Among the coumarin analogues found to be in class 3 (**6a**, **6b**, **6e**), a common feature is a short side arm consisting of 0, 1, or 3 carbons, respectively.

Finally, the analogues in class 4 only weakly reduced nerve stimulation-induced skeletal muscle contractility or actin-activated ATPase activity of skeletal myosin and therefore are poor inhibitors of skeletal and nonmuscle myosin IIB. Among them, only **6f** has an IC_{50} value of <100 μM for cardiac myosin (Table 1), suggesting that it may be a good lead compound for cardiac selectivity.

Chemical Stability, Solubility, and Toxicity.

Drug practicality is often limited by the chemical and physical properties, including toxicity, solubility, and stability.⁴⁹ The amine side arm is important for myosin inhibition potency and selectivity for the analogues tested in this study. Since the side arm hydrophobicity could limit solubility, we measured kinetic solubilities of three key BHC compounds in pH 7.4 phosphate-buffered saline (PBS) containing 2.5% DMSO, as follows: **2**, 607 ± 2 ; **7a**, 103 ± 3 ; and **7i**, >400 μM . The benzyl group in **7a** has a deleterious effect on solubility, but its solubility/potency ratio is much higher than for **1a**.^{11,14} As expected, the presence of the polar pyridine ring side arm in **7i** leads to higher aqueous (aq) solubility.

Most of the analogues are Schiff bases, which exist primarily as enamine tautomers in both polar and nonpolar solvents. The imine functional group has the potential to undergo hydrolysis under aqueous conditions. Unconjugated imines and enamines can hydrolyze under these conditions, but BHC and its analogues are stabilized by electron delocalization. To assess hydrolytic stability, a 100 μM solution of **7i** in 99:1 (v/v) PBS/methanol was monitored by UV–visible absorption spectroscopy during storage under ambient room

conditions (light and temperature). The half-time for hydrolysis of **7i** in 99:1 (v/v) pH 7.4 PBS/methanol was found to be 3.5 ± 0.5 days (see the Supporting Information). This is much longer than typical half-lives of the drugs in the body, so we conclude that the stabilities of the imine functionality in these analogues are adequate for clinical application.

There is very little toxicity data on the BHC compounds that were previously reported in the literature, but what is available is very encouraging. In particular, the cytotoxicities of **6b**, **6j**, and **7a** in the promyelocytic HL-60 leukemia cells have been reported as 408, 590, and 801 μM , respectively.⁴⁰ This suggests that the drugs developed from this structural class may have acceptable toxicities.

SUMMARY AND CONCLUSIONS

We have used a combination of solution ATPase and *in vitro* actin-sliding assays, *ex vivo* skeletal neuromuscular physiology, and *in silico* molecular docking to provide insights into the molecular mechanisms of inhibition and binding, as well as selectivity of BHC and a series of 34 analogues for three abundant isoforms of myosin II found in cardiac and skeletal muscles and in nonmuscle cells.

We provide several lines of evidence to suggest that BHC and analogues bind to the same site as blebbistatin and have similar mechanisms of action. First, blebbistatin and several BHC analogues inhibit the steady-state actin-activated ATPase activity of both skeletal and cardiac myosin with similar potency and selectivity. Since this activity represents the rate-limiting step in the ATPase cycle of myosin II, BHC analogues most likely stabilize the actin-unbound myosin-ADP-P_i state, as has been previously shown for blebbistatin. Second, we show that BHC, like blebbistatin, inhibits the actin-sliding velocity as measured in an *in vitro* motility assay. Third, an analogue of BHC can compete with blebbistatin for equilibrium binding to skeletal myosin. Finally, docking studies with blebbistatin and BHC analogues using a template structure and two different homology models suggest that most BHC analogues, like blebbistatin, bind within the large cleft between the upper and lower 50 kDa subdomains of the motor domain. Details of the predicted binding geometries show that similar residues stabilize the binding of BHC and blebbistatin. However, since the binding site is relatively conserved among the myosin II isoforms studied here, modeling did not give significant insights into isoform selectivity.

The SAR and modeling studies have identified several regions of the BHC scaffold that tune the inhibitory potencies and therefore confer selectivity for skeletal, cardiac, or nonmuscle myosins. A unique aspect of this study is an *ex vivo* skeletal muscle preparation that allows simultaneous evaluation effects of the compounds on (1) neurotransmission, which depends on both the pre- and postsynaptic roles of nonmuscle myosin II,^{47,48,50,51} and (2) contractility, which depends upon muscle myosin. A combination of these data with the IC₅₀ values for ATPase inhibition of the skeletal and cardiac muscles allows further SAR to emerge. A key finding is that three analogues with shorter side arms (1–3 carbons) with IC₅₀ 100 μM for skeletal and cardiac myosin ATPase activity blocked neurotransmission, suggesting that they are selective for nonmuscle myosin IIB. This same pattern was also found for several analogues with the quinolinone scaffold with shorter side arms, suggesting

that nonmuscle myosin IIB can accommodate nitrogen *versus* oxygen within the ring. More quantitative information about relative potencies will be addressed in future studies with purified nonmuscle myosin IIB.

All analogues with branched aliphatic side arms were poor inhibitors of both skeletal and cardiac myosins, but branched side arms with more than four carbons were not investigated. Adding more than three carbons to the linear aliphatic side arms was required for potency for both cardiac and skeletal myosins. Incorporation of an aromatic ring improved the potency further. Replacing benzene with pyridine reduced potency, but 4-pyridylmethyl analogue **7i** had the highest selectivity (12-fold) for inhibition of skeletal *versus* cardiac myosin. Importantly, **7i** lacked potency for nonmuscle myosin IIB. Therefore, **7i** appears to be a promising candidate to relax the skeletal muscle with less effect to slow the heartbeat or to affect the many functions in the nonmuscle cells mediated by nonmuscle myosin IIB, most importantly in the peripheral and central nervous systems.⁵²

While pharmacological, genetic, and immunohistochemical evidence^{47,50,53} suggests that inhibition of NMIIIB, rather than NMIIA or NMIIC, likely underlies the effects of blebbistatin and BHC analogues on neurotransmission observed in this study, we cannot rule out effects on other nonmuscle myosins such as myosin VI, which also regulates synaptic transmission and is sensitive to blebbistatin.⁵⁴

The models of the BHC analogues bound to myosin at the blebbistatin site provide insights that are generally consistent with the SAR trends summarized above. Importantly, the side arms of the BHC analogues appear to interact with the hydrophobic residues lining an extension of the binding pocket, which accommodates the longest side arm length of **6i** (six carbons) and might accommodate the longer side chains in new analogues. Future drug-design efforts could focus on tailoring aliphatic and aromatic ring hydrophobic interactions in the side arm binding region to enhance potency and selectivity and also on adding groups of moderate polarity to adjust the physical properties, such as solubility. Available data on solubility, stability, and toxicity suggest that the BHC scaffold may be used to develop clinical candidates.

Clinical Relevance.

BHC (**2**) and blebbistatin (**1a**) have comparable mechanisms of action, leading to the question of which scaffold has greater promise for developing clinical candidates. Both compounds inhibit skeletal and cardiac myosin II ATPase activity with similar overall potencies. However, based on our physiological studies, BHC does not inhibit nonmuscle myosin II, whereas blebbistatin does.^{55,56} To date, we have found one analogue (**7i**) that shows further improvement in this property with similar potency to BHC, but with much better selectivity for skeletal myosin. These data, together with a recent study, which showed that specifically designed analogues of blebbistatin failed to retain myosin inhibitory function (**13**), suggest that BHC represents a valuable tool for the synthesis of myosin II inhibitors.

EXPERIMENTAL SECTION

Ethical Approval and Use of Mice.

Adult wild-type C57Bl6 mice of each sex (8–12 weeks of age) were killed by isoflurane inhalation and cervical dislocation, in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Nevada, Reno.

Biological Evaluation: Reagents.

(–)-Blebbistatin (CAS 856925–71-8) was purchased from Calbiochem, ATP (A3377); buffer components were from Sigma. The assay buffer was 10 mM 3-morpholinopropane-1-sulfonic acid (MOPS), pH 7.0, 50 mM NaCl, 0.1 mM ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid (EGTA), 5 mM MgCl₂, and 1 mM dithiothreitol (DTT). Dilution series of all analogues were prepared in DMSO from 25 mM stock solution, which were protected from light and stored at –20 °C.

Proteins.

Skeletal muscle myosin was purified⁵⁷ from fresh rabbit psoas muscle and cardiac myosin was purified⁵⁸ from fresh bovine left ventricles. Both types of myosin were stored at –20 °C in 50% aq glycerol and were shown by urea gel electrophoresis to be unphosphorylated (data not shown). The S1 head subfragment of skeletal myosin was prepared by chymotryptic digestion⁵⁹ and stored in 4 mg mL^{–1} aq sucrose at –80 °C. Actin was purified⁶⁰ from the rabbit psoas muscle and stored on ice as F-actin.

Steady-State Actin-Activated ATPase Assays.

ATPase assays (250 μ L) were performed at 30 °C in assay buffer with a final DMSO concentration of 2% (v/v). This level of DMSO had no detectable effect on ATPase activity or motility (data not shown). Dilutions of compounds in DMSO (5 μ L) were added to a series of 8 epi tubes (1 mL) with final concentrations ranging from 0.001 to 30 μ M. Since acto-myosin is not a soluble protein system, higher concentrations of analogues were avoided to ensure against nonspecific protein aggregation. A premix was prepared that contained sufficient actin and ATP in assay buffer to give final concentrations of 32 μ M and 2 mM, respectively. Using a positive displacement pipette, 195 μ L of premix was added and mixed to evenly disperse the DMSO. After temperature equilibration in a heating block, myosin (50 μ L) was added at $t = 0$ to a final concentration of 0.1 (skeletal) or 0.5 (cardiac) mg mL^{–1}, followed by extensive mixing. At 6, 12, 18, and 24 min, 50 μ L of the aliquots was quenched into 50 μ L of 5% aq sodium dodecyl sulfate (Sigma, L3771) that had been preloaded into a 96-well microplate using a liquid handler interfaced to a microplate reader. MeOH (20 μ L) was then added by hand to remove bubbles.

Phosphate was determined spectrophotometrically by a modification of the method of Chifflet et al.⁶¹ as described in detail.²⁵ A discontinuous assay was required because full length myosins form insoluble filaments under the near physiological conditions of the assay buffer. Light scattering and inhomogeneity lead to irreproducible data with continuous luminescence or fluorescence-based assays in a microplate format.

Analysis of ATPase Data.

Time courses of P_i production typically gave changes in absorbance (850 nm) ranging from 0.2 to 1.5 and were fit to a line. The slopes for each titration ($N = 1$) were normalized to the highest value and N data sets were plotted together prior to fitting to a four-parameter logistics equation without weighting. Reported errors (Table 1) represent the standard error of the fit. Titrations with **1b**, which is highly soluble and gave no detectable abnormal aggregation, showed that ATPase activity of both cardiac and skeletal myosin activity diminished to ~2% of maximum at 500 μM . Therefore, minimum activity was fixed to 2% for the logistics fitting, whereas maximal activity, slope, and IC_{50} values were left to float. For analogues with $\text{IC}_{50} = 100 \mu\text{M}$, the average slopes for cardiac and skeletal myosin were 0.9 ± 0.2 and 0.7 ± 0.1 , respectively.

In vitro Motility Assays.

Assays with 100 $\mu\text{g mL}^{-1}$ skeletal myosin were performed at 30 °C as previously described²⁵ with the following modifications. Final conditions during the assay were assay buffer with 2 mM ATP, BHC (final DMSO 2%), 0.4% methylcellulose, and an oxygen scavenging system consisting of 6 U mL^{-1} pyranose oxidase (Sigma, P4234), 80–200 U mL^{-1} catalase (Sigma, C1345), and glucose (40 mM). Importantly, none of the assay constituents contained azide, which strongly inhibits catalase.^{62,63} The slides were incubated for 1 min at 30 °C on a slide warmer to decrease dissolved O_2 prior to imaging to reduce actin shredding and stuck filaments. The images were collected with a Nikon TE2000 inverted microscope, 100 $\times$ plan apo Nikon objective (NA = 1.45) with a heating collar set to maintain the flow cell at 30 °C, and a 1.5 $\times$ tube lens. Total internal reflection excitation was controlled with a Cairn Optics OptoTIRF module. Actin was imaged with a 532 nm excitation laser (QiOptiq) and a 565/40 bandpass emission filter (Chroma). An Andor iXon 888 Ultra camera was controlled using Metamorph (Molecular Devices). Exposure times were set from 0.1 to 1 s so that the filaments moved at least ~5–10 pixels per frame to avoid oversampling, which leads to falsely high velocities. The field of view was 44.4 μm^2 (512 pixels²) at 86.7 nm pixel⁻¹. Three 45–60 s movies were collected per slide with three slides per inhibitor concentration. The movies were analyzed with FAST software^{64,65} using a 20% filtering cutoff and five-frame minimum. The filaments shorter than 1 μm were excluded. Replicate slide data were averaged $\pm$ standard deviation to give each point in Figure 5, which represent on average 340 individual actin trajectories.

Homology Modeling and Docking.

The *D. discoideum* myosin II motor domain co-crystallized with **1** (PDB: 1yv3)⁴⁴ was used as the template to build a homology model of the bovine cardiac β -myosin heavy chain (UniProt Q9BE39 (MYH7_BOVIN; gene: MYH7)) and rabbit skeletal myosin heavy chain (UniProt Q9GJP9 (Q9GJP9_RA-BIT; gene: MYH13)) using SWISS-MODEL.⁶⁶ AutoDock Vina⁶⁷ was used for all docking. To validate the docking method, **1** was docked onto 1yv3 (without **1**) and the entire apo-cardiac myosin structure. The highest scoring poses (out of 7) for each structure were consistent with binding for **1** in 1yv3. This was also true for **2** using the same criteria. To dock other analogues, a 40 Å³ search grid was used instead of the entire

structure. The center point for docking was placed at the nitrogen of Ala463 (Ser456 in 1yv3) of cardiac myosin because it is known to interact with **1**.

Physiological Methods.

After euthanizing mice with CO₂ inhalation, the diaphragms of the adult mice were dissected, pinned on a Sylgard-coated dish and placed in oxygenated Kreb's Ringers solution, and allowed to equilibrate for 3 min. For stimulation, the phrenic nerve was drawn into a suction electrode driven by an SD9 square pulse stimulator (suprathreshold pulses at 8–10 V, pulse width of 0.1 ms) as described previously.²² Drug stocks (25 mM in DMSO) were dissolved as follows: for 100 μ M, a mixture of DMSO (8 μ L) and stock (32 μ L) was added into 1 mL of Kreb's Ringers solution, before adding to dish contents (7 mL). For this dose, the DMSO concentration was 40 μ L/8000 μ L or 0.5%. For experiments at 200 or 300 μ M, the final DMSO was accordingly 1.0 and 1.5%. We performed control studies in 0.5–1.5% DMSO in the absence of drugs and failed to observe any deleterious effect on contractility. For electrophysiological recording, we impaled the muscle cells near the endplate region with pipettes with tip resistances of ~30–60 M Ω .²²

Kinetic Solubility.

The following procedure⁶⁸ was used to determine kinetic solubility. Solutions of each compound with various starting concentrations in 2.5% DMSO with a final volume of 100 μ L were prepared in quadruplicate by serial dilution on sterile 96-well microplates. Using a micropipette, the appropriate amount of a 20 mM DMSO stock solution for each compound tested was added into a vial containing 858 μ L of PBS buffer (pH = 7.4) and the appropriate amount of DMSO to keep the DMSO concentration constant. A second starting solution with a lower concentration of the compound was prepared in the same manner. Mixing with a micropipette, serial dilutions were performed from the starting solution with each well containing a final volume of 100 μ L. Each plate was placed in a Nepheloskan Ascent nephelometer with the lid off, shaken for 2 min, incubated for 1 h at 37 °C, and shaken again for 3 min before measuring each well. The raw data was adjusted by subtracting the average blank value and analyzed using Microsoft Excel.

Chemistry: General Methods.

The reagents and solvents purchased from Acros Organics, Sigma-Aldrich, or Fisher Scientific were of ACS reagent grade or better and were used without purification. All reactions were performed under air unless otherwise noted. "Overnight" periods are *ca.* 16 h. Flash column chromatography was performed on a Yamazen AKROS flash chromatograph using prepackaged neutral silica (40 μ m particle, 60 Å pore size) or neutral aluminum oxide (40 μ m particle, 60 Å pore size). The melting points were measured on a Thomas-Hoover capillary melting point apparatus and are uncorrected. The ¹H NMR (400 or 500 MHz) and ¹³C NMR (101 or 126 MHz) spectra were acquired on a Varian 400 or Varian Unity +500 spectrometer. All chemical shifts (δ) are reported in parts per million (ppm) units relative to solvent resonance, as follows: ¹H, CDCl₃/tetramethylsilane (TMS) = 0.00, DMSO-*d*₆/TMS = 0.00; ¹³C, CDCl₃ = 77.16, DMSO-*d*₆ = 39.52. The infrared (IR) spectra were recorded neatly on a Nicolet 6700 FTIR spectrometer. The low-resolution mass spectra (MS) were

acquired on a Waters Micromass ZQ electrospray ionization quadrupole mass spectrometer with positive ion detection (capillary voltage = 3.5 kV). The UV–visible spectra (UV/vis) were recorded on a Shimadzu UV-2550 UV–visible spectrophotometer; the molar extinction coefficients (ϵ , $M^{-1} \text{ cm}^{-1}$) were determined by the Beer–Lambert law plots of four to five data points in the absorbance range of 0.2–0.8 au. The samples for elemental analysis were dried at 78 °C (0.1 mm) for 2 days and microanalysis was performed by NuMega Resonance Labs, Inc. The purities of all samples submitted for biological testing were greater than 95%, as shown by combustion microanalysis and ^1H NMR spectroscopy.

3-Acetyl-4-hydroxy-2H-chromen-2-one (4).²⁹—A solution of 2.1 g (13 mmol) of 4-hydroxy-2H-chromen-2-one (**3**) in 10 mL of glacial acetic acid was stirred as 3.5 mL (37 mmol) of anhydrous POCl_3 was added in one portion. The resulting solution was stirred and boiled under reflux for 45 min, then cooled to room temperature. The resulting precipitate was collected by filtration and recrystallized from ethanol, yielding 1.60 g (60%) of **4** as transparent crystals, mp 131–133 °C (lit.^{28,29} 135, 133–135 °C). ^1H NMR (500 MHz, CDCl_3) δ 8.07 (dd, 7.9, 1.7 Hz, 1H, H5), 7.70 (ddd, 8.2, 7.3, 1.7 Hz, 1H, H7), 7.36–7.29 (m, 2H, H6,8), 2.79 (s, 3H, CH_3). ^{13}C NMR (101 MHz, CDCl_3) δ 206.2, 178.9, 160.2, 154.9, 136.3, 125.8, 124.6, 117.2, 115.4, 101.6, 30.3.

Schiff bases **2**, **6a–k**, **7a–i**, **10a**, **10b**, and **12a–i** were synthesized from primary amines^{30,31} by reaction of 3-acyl-4-hydroxycoumarins with ammonium salts and purified by one of the following three methods, unless stated otherwise. A 0.2–0.8 M solution of **4** and 1.1–2 equiv of an amine in the stated solvent was stirred and boiled under reflux 4–16 h, then cooled to room temperature. In method A, the resulting precipitate was collected by filtration and recrystallized from ethanol, in method B, the solution was concentrated to dryness by rotary evaporation and the product was purified by recrystallization from ethanol, and in method C, the solution was concentrated to dryness by rotary evaporation and the product was purified by flash chromatography on silica, eluting with the described solvent(s). Note: While the names of these compounds describe them conventionally as imines, the NMR spectra show that all are enamines in solution, except for oxime **7e** and hydrazone **7f**. The ^1H NMR spectra are assigned accordingly.

3-(N-Butylethanimidoyl)-4-hydroxy-2H-chromen-2-one (2).²¹—Following method A, condensation of 0.32 g (1.6 mmol) of **4** with butylamine in ethanol gave 0.39 g (78%) of **2** as white crystals, mp 105 °C (lit.²⁶ 105–107 °C). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 13.77 (s, 1H, NH), 7.95 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.63 (ddd, 8.3, 7.2, 1.7 Hz, 1H, H7), 7.28 (m, 2H, H6,8), 3.61 (t, 7.1 Hz, 2H, NCH_2), 2.66 (s, 3H, NCCH_3), 1.66 (quint, 7.2, 2H, NCH_2CH_2), 1.43 (sext, 7.6 Hz, 2H, CH_2CH_3), 0.95 (t, 7.3 Hz, 3H, CH_2CH_3). ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 179.6, 176.1, 161.8, 153.0, 133.9, 125.6, 123.6, 120.2, 116.29, 96.0, 43.5, 30.3, 19.5, 18.3, 13.5. IR (cm^{-1}) 2953 (w), 2929 (w), 2871 (w), 1695 (s), 1596 (s), 1459 (s). MS m/z 260 (MH^+). Anal. calcd for $\text{C}_{15}\text{H}_{17}\text{NO}_3$: C, 69.48; H, 6.61; N, 5.40. Found: C, 69.14; H, 7.00; N, 5.65. UV/vis (DMSO) λ_{max} , nm (ϵ): 325 (1.89×10^4).

3-Ethanimidoyl-4-hydroxy-3-2H-chromen-2-one (6a).—A solution of 0.48 g (2.4 mmol) of **4** and 0.20 g (2.6 mmol) of ammonium acetate in 10 mL of ethanol was stirred

under a N₂ atmosphere and boiled under reflux overnight, cooled to room temperature, diluted with 15 mL of water, and extracted with dichloromethane (DCM) (3 × 15 mL). The combined extracts were dried (Na₂SO₄), filtered, and concentrated to dryness by rotary evaporation. The residue was purified by flash chromatography on silica, eluting with 7:3 (v/v) hexanes/EtOAc, yielding 0.19 g (40%) of **6a** as white crystals, mp 234 °C (lit.^{31,36,69,70} 234.5–234.9, 236.3–237.4, 221–225 °C). ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.99 (s, 1H, NH), 9.96 (s, 1H, NH), 7.90 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.59 (ddd, 8.2, 7.2, 1.7 Hz, 1H, H7), 7.24 (m, 2H, H6,8), 2.55 (s, 3H, CH₃). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 179.6, 177.3, 153.1, 134.0, 125.7, 123.6, 120.4, 116.3, 110.4, 95.7, 24.5. IR (cm⁻¹) 3252 (m, br), 2981 (m), 2361 (m), 1684 (m), 1606 (s), 1567 (m), 1453 (s). MS *m/z* 204 (MH⁺). Anal. calcd for C₁₁H₉NO₃: C, 65.02; H, 4.46; N, 6.89. Found: C, 64.78; H, 5.09; N, 6.99. UV/vis (MeOH) λ_{max}, nm (ε): 317 (1.67 × 10⁴).

4-Hydroxy-3-(N-methylethanimidoyl)-2H-chromen-2-one (6b).—Following method A, condensation of 1.0 g (5.1 mmol) of **4** with methylamine in methanol gave 0.81 g (59%) of **6b** as white crystals, mp 180 °C (lit.⁴⁰ 181–183 °C). ¹H NMR (400 MHz, DMSO-*d*₆) δ 13.44 (s, 1H, NH), 7.95 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.63 (m, 1H, H7), 7.28 (m, 2H, H6,8), 3.23 (d, 4.8 Hz, 3H, NCH₃), 2.65 (s, 3H, NCCH₃). ¹³C NMR (126 MHz, CDCl₃) δ 181.4, 177.8, 162.81, 133.9, 126.1, 123.6, 120.5, 116.6, 110.1, 97.3, 30.9, 18.5. IR (cm⁻¹) 2360 (w), 1692 (m), 1601 (s), 1568 (s), 1461 (m). MS *m/z* 218 (MH⁺). Anal. calcd for C₁₂H₁₁NO₃: C, 66.35; H, 5.10; N, 6.45. Found: C, 66.39; H, 5.49; N, 6.43. UV/vis (DMSO) λ_{max}, nm (ε): 324 (1.80 × 10⁴); (MeOH) λ_{max}, nm (ε): 316 (1.69 × 10⁴).

3-(N-Ethylethanimidoyl)-4-hydroxy-2H-chromen-2-one (6c).—A solution of 1.0 g (5.0 mmol) of **4** and 0.80 g (7.6 mmol) of ethylammonium acetate in 10 mL of ethanol was stirred and boiled under reflux overnight, cooled to room temperature, diluted with 20 mL of chloroform, washed with water (3 × 20 mL), dried (Na₂SO₄), filtered, and concentrated to dryness by rotary evaporation. Recrystallization from ethanol gave 0.77 g (53%) of **6c** as white crystals, mp 145 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 13.67 (s, 1H, NH), 7.95 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.63 (ddd, 8.2, 7.2, 1.7 Hz, 1H, H7), 7.28 (m, 2H, H6,8), 3.64 (qd, 7.3, 5.4 Hz, 2H, CH₂), 2.66 (s, 3H, NCCH₃), 1.29 (t, 7.3 Hz, 3H, CH₂CH₃). ¹³C NMR (126 MHz, CDCl₃) δ 181.2, 176.2, 162.6, 153.6, 133.6, 125.8, 123.5, 120.5, 116.5, 96.88, 39.2, 18.5, 14.3. IR (cm⁻¹) 2979 (w), 2360 (w), 1699 (m), 1592 (m), 1457 (s). MS *m/z* 232 (MH⁺). Anal. calcd for C₁₃H₁₃NO₃: C, 67.52; H, 5.67; N, 6.06. Found: C, 67.41; H, 6.07; N, 6.31. UV/vis (MeOH) λ_{max}, nm (ε): 317 (1.65 × 10⁴).

4-Hydroxy-3-(N-propylethanimidoyl)-2H-chromen-2-one (6d).—Following method A, condensation of 0.77 g (3.8 mmol) of **4** with propylamine in methanol gave 0.84 g (73%) of **6d** as white crystals, mp 128 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 13.78 (s, 1H, NH), 7.95 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.63 (ddd, 8.2, 7.2, 1.7 Hz, 1H, H7), 7.28 (m, 2H, H6,8), 3.58 (td, 7.0, 5.4 Hz, 2H, NCH₂), 2.66 (s, 3H, NCCH₃), 1.70 (sext, 7.3 Hz, 2H, CH₂CH₃), 1.00 (t, 7.4 Hz, 3H, CH₂CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 181.2, 176.3, 162.7, 153.5, 133.6, 125.8, 123.4, 120.5, 116.4, 96.9, 46.0, 22.3, 18.6, 11.4. IR (cm⁻¹) 2964 (w), 2879 (w), 2360 (w), 1682 (m), 1604 (s), 1571 (s), 1466 (m). MS *m/z* 246 (MH⁺). Anal.

calcd for $C_{14}H_{15}NO_3$: C, 68.56; H, 6.16; N, 5.71. Found: C, 68.27; H, 6.54; N, 5.66. UV/vis (MeOH) $\lambda_{\max}$, nm (ϵ): 319 (1.71×10^4).

4-Hydroxy-3-(N-isopropylethanimidoyl)-2H-chromen-2-one (6e).—A solution of 0.48 g (2.3 mmol) of **4** and 0.80 g (2.5 mmol) of isopropylammonium acetate in 5 mL of ethanol was stirred and boiled under reflux overnight, cooled to room temperature, diluted with 20 mL of DCM, washed with water (3×15 mL), dried (Na_2SO_4), filtered, and concentrated to dryness by rotary evaporation. Recrystallization from ethanol gave 0.41 g (57%) of **6e** as white crystals, mp 107 °C. 1H NMR (400 MHz, DMSO- d_6) δ 13.85 (s, 1H, NH), 7.91 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.59 (ddd, 8.2, 7.2, 1.7 Hz, 1H, H7), 7.24 (m, 2H, H6,8), 4.23 (m, 1H, CH), 2.64 (s, 3H, $NCCH_3$), 1.28 (d, 6.4 Hz, 6H, $CH(CH_3)_2$). ^{13}C NMR (101 MHz, DMSO- d_6) δ 179.7, 174.8, 161.8, 153.0, 133.9, 125.6, 123.6, 120.1, 116.2, 95.8, 45.9, 22.3, 18.3. IR (cm^{-1}) 2973 (w), 1708 (s), 1596 (s), 1569 (s), 1463 (s). MS m/z 146 (MH^+). Anal. calcd for $C_{14}H_{15}NO_3$: C, 68.56; H, 6.16; N, 5.71. Found: C, 68.16; H, 6.48; N, 5.88. UV/vis (MeOH) $\lambda_{\max}$, nm (ϵ): 316 (1.66×10^4).

4-Hydroxy-3-(N-isobutylethanimidoyl)-2H-chromen-2-one (6f).—Following method B, condensation of 0.40 g (2.0 mmol) of **4** with isobutylamine (neat) gave 0.64 g (67%) of **6f** as white crystals, mp 80 °C. 1H NMR (400 MHz, DMSO- d_6) δ 13.86 (s, 1H, NH), 7.92 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.59 (ddd, 8.2, 7.2, 1.7 Hz, 1H, H7), 7.25 (m, 2H, H6,8), 3.43 (d, 6.7 Hz, 2H, CH_2), 2.62 (s, 3H, $NCCH_3$), 1.98 (m, 1H, CH), 0.97 (d, 6.7 Hz, 6H, $CH(CH_3)_2$). ^{13}C NMR (101 MHz, DMSO- d_6) δ 179.7, 176.2, 161.8, 153.0, 133.9, 125.6, 123.5, 120.1, 116.2, 96.1, 50.9, 27.7, 19.7, 18.3. IR (cm^{-1}) 2959 (w), 2874 (w), 1694 (s), 1602 (s), 1572 (m), 1483 (w), 1465 (s). MS m/z 260 (MH^+). Anal. calcd for $C_{15}H_{17}NO_3$: C, 69.48; H, 6.61; N, 5.40. Found: C, 69.15; H, 7.01; N, 5.40. UV/vis (MeOH) $\lambda_{\max}$, nm (ϵ): 318 (1.56×10^4).

3-(N-sec-Butylethanimidoyl)-4-hydroxy-2H-chromen-2-one (6g).—Following method A, condensation of 0.77 g (3.8 mmol) of **4** with *sec*-butylamine in methanol gave 0.60 g (49%) of **6g** as white crystals, mp 87 °C. 1H NMR (400 MHz, DMSO- d_6) δ 13.89 (s, 1H, NH), 7.91 (ddd, 7.9, 1.7, 0.5 Hz, 1H, H5), 7.59 (ddd, 8.2, 7.2, 1.7 Hz, 1H, H7), 7.28 (m, 2H, H6,8), 4.08 (m, 1H, CH), 2.68 (s, 3H, $NCCH_3$), 1.66 (m, 2H, CH_2), 1.28 (d, 6.5 Hz, 3H, $CHCH_3$), 0.95 (t, 7.4 Hz, 3H, CH_2CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ 181.3, 175.2, 162.9, 153.7, 133.7, 125.9, 123.5, 120.6, 116.5, 96.9, 52.0, 29.9, 20.7, 18.8, 10.3. IR (cm^{-1}) 2966 (w), 2926 (w), 2853 (w), 2360 (w), 1696 (m), 1594 (s), 1566 (s), 1459 (s). MS m/z 260 (MH^+). Anal. calcd for $C_{15}H_{17}NO_3$: C, 69.48; H, 6.61; N, 5.40. Found: C, 69.19; H, 6.97; N, 5.34. UV/vis (MeOH) $\lambda_{\max}$, nm (ϵ): 318 (1.76×10^4).

4-Hydroxy-3-(N-pentylethanimidoyl)-2H-chromen-2-one (6h).—Following method B, condensation of 0.65 g (3.2 mmol) of **4** with pentylamine in ethanol gave 0.70 g (65%) of **6h** as white crystals, mp 101 °C. 1H NMR (400 MHz, DMSO- d_6) δ 13.76 (s, 1H, NH), 7.95 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.63 (ddd, 8.3, 7.2, 1.7 Hz, 1H, H7), 7.28 (m, 2H, H6,8), 3.60 (td, 7.0, 5.4 Hz, 2H, NCH_2), 2.66 (s, 3H, $NCCH_3$), 1.68 (quint, 7.3 Hz, 1H, NCH_2CH_2), 1.37 (m, 4H, $(CH_2)_2CH_3$), 0.90 (t, 7.1, 3H, CH_2CH_3). ^{13}C NMR (101 MHz, DMSO- d_6) δ 179.6, 176.0, 161.8, 153.2, 133.9, 125.6, 123.6, 120.1, 116.2, 96.0, 43.8, 28.4,

28.0, 21.7, 18.2, 13.8. IR (cm^{-1}) 2954 (w), 2928 (w), 2857 (w), 1687 (s), 1605 (s), 1571 (m), 1460 (s). MS m/z 274 (MH^+). Anal. calcd for $\text{C}_{16}\text{H}_{19}\text{NO}_3 \cdot 0.25\text{H}_2\text{O}$: C, 69.17; H, 7.07; N, 5.04. Found: C, 69.52; H, 7.40; N, 5.36. UV/vis (MeOH) λ_{max} , nm (ϵ): 317 (1.60×10^4).

3-(N-Hexylethanimidoyl)-4-hydroxy-2H-chromen-2-one (6i).—Following method B, condensation of 0.41 g (2.0 mmol) of **4** with hexylamine in ethanol gave 0.53 g (91%) of **6i** as white crystals, mp 101–102 °C. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 13.76 (s, 1H, NH), 7.95 (dd, 7.9, 1.7 Hz, 1H, H5), 7.63 (ddd, 8.3, 7.2, 1.7 Hz, 1H, H7), 7.28 (m, 2H, H6,8), 3.60 (td, 7.1, 5.4 Hz, 2H, NCH_2), 2.65 (s, 3H, NCCH_3), 1.67 (quint, 7.1 Hz, 2H, NCH_2CH_2), 1.36 (m, 6H, $(\text{CH}_2)_3\text{CH}_3$), 0.89 (t, 7.2 Hz, 3H, CH_2CH_3). ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 179.6, 176.0, 161.8, 153.0, 133.9, 125.6, 123.6, 120.2, 116.2, 96.0, 43.8, 30.7, 28.2, 25.9, 22.0, 18.3, 13.8. IR (cm^{-1}) 2928 (m), 2860 (w), 1700 (s), 1599 (s), 1574 (s), 1459 (m). Anal. calcd for $\text{C}_{17}\text{H}_{21}\text{NO}_3$: C, 71.06; H, 7.37; N, 4.87. Found: C, 70.67; H, 7.75; N, 5.16. UV/vis (MeOH) λ_{max} , nm (ϵ): 319 (1.73×10^4).

4-Hydroxy-3-(N-(2-hydroxyethyl)ethanimidoyl)-2H-chromen-2-one (6j).—

Following method B, condensation of 1.0 g (4.9 mmol) of **4** with 2-aminoethanol in ethanol gave 1.26 g (83%) of **6j** as white crystals, mp 178 °C (lit.^{30,36,40,71,72} 183–185, 196, 157.2–159.4, 181–183 °C). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 13.77 (s, 1H, NH), 7.95 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.63 (ddd, 8.2, 7.2, 1.7 Hz, 1H, H7), 7.28 (m, 2H, H6,8), 3.68 (m, 4H, $\text{N}(\text{CH}_2)_2$), 2.67 (s, 3H, CH_3). ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 179.4, 176.2, 161.9, 153.0, 133.8, 125.6, 123.6, 120.3, 116.2, 96.0, 58.9, 46.5, 18.5. IR (cm^{-1}) 3405 (w), 2948 (w), 1360 (w), 1656 (m), 1588 (m), 1570 (m), 1486 (m), 1457 (m). MS m/z 248 (MH^+). Anal. calcd for $\text{C}_{12}\text{H}_{11}\text{NO}_4 \cdot 0.25\text{H}_2\text{O}$: C, 62.02; H, 5.41; N, 5.56. Found: C, 61.94; H, 5.34; N, 5.86. UV/vis (MeOH) λ_{max} , nm (ϵ): 317 (1.61×10^4).

4-Hydroxy-3-(N-(3-hydroxypropyl)ethanimidoyl)-2H-chromen-2-one (6k).—

Following method B, condensation of 0.32 g (1.5 mmol) of **4** with 3-amino-1-propanol in ethanol gave 0.38 g (75%) of **6k** as white crystals, mp 139 °C (lit.⁷³ 154 °C). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 13.74 (s, 1H, NH), 7.95 (ddd, 7.9, 1.8, 0.5 Hz, 1H, H5), 7.63 (ddd, 8.2, 7.2, 1.7 Hz, 1H, H7), 7.28 (m, 2H, H6,8), 3.67 (t, 6.9 Hz, 2H, CH_2OH), 3.54 (t, 6.0 Hz, 2H, NCH_2), 2.67 (s, 3H, CH_3), 1.82 (quint, 6.4, 2H, NCH_2CH_2). ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 179.51, 176.10, 161.86, 153.03, 133.91, 125.60, 123.58, 120.19, 116.18, 96.02, 57.70, 41.07, 31.43, 18.19. IR (cm^{-1}) 3481 (m), 2934 (w), 2887 (w), 2361 (w), 1672 (m), 1610 (m), 1573 (m), 1456 (m). MS m/z 262 (MH^+). Anal. calcd for $\text{C}_{14}\text{H}_{15}\text{NO}_4$: C, 64.36; H, 5.79; N, 5.36. Found: C, 64.46; H, 5.74; N, 5.70. UV/vis (MeOH) λ_{max} , nm (ϵ): 318 (1.76×10^4).

3-(N-Benzylethanimidoyl)-4-hydroxy-2H-chromen-2-one (7a).—Following method B, condensation of 0.42 g (2.0 mmol) of **4** with benzylamine in ethanol gave 0.53 g (72%) of **7a** as white crystals, mp 162 °C (lit.^{40,74} 162–163, 151–152 °C). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 13.99 (s, 1H, NH), 7.93 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.63 (ddd, 8.2, 7.3, 1.7 Hz, 1H, H7), 7.48–7.35 (m, 5H, Ph), 7.32–7.24 (m, 2H, H6,8), 4.89 (d, 5.5 Hz, 2H, CH_2), 2.72 (s, 3H, CH_3). ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 179.7, 176.3, 161.8, 153.0, 135.9, 134.0, 129.0, 128.0, 127.7, 125.6, 123.6, 120.1, 116.21, 96.3, 47.5, 18.6. IR (cm^{-1}) 1695

(m), 1610 (m), 1570 (s), 1463 (s). MS m/z 294 (MH^+). Anal. calcd for $C_{18}H_{15}NO_3$: C, 73.71; H, 5.15; N, 4.78. Found: C, 73.71; H, 5.52; N, 5.18. UV/vis (MeOH) λ_{max} , nm (ϵ): 317 (1.77×10^4).

4-Hydroxy-3-(N-(*o*-methylbenzyl)ethanimidoyl)-2H-chromen-2-one (7b).—

Following method B, condensation of 0.51 g (2.5 mmol) of **4** with *o*-methylbenzylamine in ethanol gave 0.55 g (72%) of **7b** as white crystals, mp 158–159 °C. 1H NMR (400 MHz, DMSO- d_6) δ 13.92 (s, 1H, NH), 7.92 (ddd, 7.8, 1.8, 0.5 Hz, 1H, H5), 7.63 (ddd, 8.1, 7.2, 1.7 Hz, 1H, H7), 7.28 (m, 6H, H6,8, tolyl), 4.87 (d, 4.3 Hz, 2H, CH_2), 2.74 (s, 3H, $NCCH_3$), 2.36 (s, 3H, $ArCH_3$). ^{13}C NMR (101 MHz, DMSO- d_6) δ 179.8, 176.5, 161.8, 153.0, 136.2, 134.1, 133.0, 130.6, 128.2, 128.1, 126.4, 125.7, 123.6, 120.1, 116.2, 96.3, 45.9, 18.6, 18.5. IR (cm^{-1}) 2863 (w), 1691 (s), 1609 (s), 1572 (s), 1464 (s). MS m/z 308 (MH^+). Anal. calcd for $C_{19}H_{17}NO_3 \cdot 0.25H_2O$: C, 73.18; H, 5.66; N, 4.49. Found: C, 73.05; H, 5.43; N, 4.77. UV/vis (MeOH) λ_{max} , nm (ϵ): 320 (1.88×10^4).

4-Hydroxy-3-(N-(*m*-methylbenzyl)ethanimidoyl)-2H-chromen-2-one (7c).—

Following method B, condensation of 0.51 g (2.5 mmol) of **4** with *m*-methylbenzylamine in ethanol gave 0.63 g (82%) of **7c** as white crystals, mp 154–155 °C. 1H NMR (400 MHz, DMSO- d_6) δ 13.96 (s, 1H, NH), 7.93 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.63 (ddd, 8.2, 7.2, 1.7 Hz, 1H, H7), 7.26 (m, 6H, H6,8, tolyl), 4.84 (s, 3H, CH_2), 2.72 (s, 3H, $NCCH_3$), 2.33 (s, 3H, $ArCH_3$). ^{13}C NMR (101 MHz, DMSO- d_6) δ 179.7, 176.3, 161.8, 153.0, 138.2, 135.8, 134.0, 128.9, 128.6, 128.3, 125.6, 124.8, 123.6, 120.1, 116.2, 96.3, 47.5, 21.0, 18.6. MS m/z 308 (MH^+). IR (cm^{-1}) 2919 (w), 1691 (m), 1612 (m), 1568 (m), 1466 (m). Anal. calcd for $C_{19}H_{17}NO_3 \cdot 0.25H_2O$: C, 73.18; H, 5.66; N, 4.49. Found: C, 73.23; H, 5.41; N, 4.69. UV/vis (MeOH) λ_{max} , nm (ϵ): 320 (1.96×10^4).

4-Hydroxy-3-(N-(*p*-methylbenzyl)ethanimidoyl)-2H-chromen-2-one (7d).—

Following method C, 0.52 g (2.5 mmol) of **4** was condensed with *p*-methylbenzylamine in ethanol. Elution with 1:1 (v/v) hexanes/EtOAc gave 0.51 g (65%) of **7d** as white crystals, mp 142–143 °C. 1H NMR (400 MHz, DMSO- d_6) δ 13.94 (s, 1H, NH), 7.92 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.63 (ddd, 8.2, 7.2, 1.7 Hz, 1H, H7), 7.28 (m, 6H, H6,8, tolyl), 4.83 (d, 5.4 Hz, 2H, CH_2), 2.71 (s, 3H, $NCCH_3$), 2.33 (s, 3H, $ArCH_3$). ^{13}C NMR (101 MHz, DMSO- d_6) δ 179.7, 176.2, 161.8, 153.0, 137.3, 134.0, 132.8, 129.5, 127.8, 125.6, 123.6, 120.1, 116.2, 96.3, 47.3, 20.7, 18.6. IR (cm^{-1}) 2917 (w), 1691 (s), 1610 (s), 1570 (s), 1516 (w), 1463 (m). Anal. calcd for $C_{19}H_{17}NO_3$: C, 74.25; H, 5.58; N, 4.56. Found: C, 74.43; H, 5.40; N, 4.90. UV/vis (MeOH) λ_{max} , nm (ϵ): 320 (1.82×10^4).

3-(N-Benzyloxyethanimidoyl)-4-hydroxy-2H-chromen-2-one (7e).—

Following method C, 0.50 g (2.5 mmol) of **4** was condensed with *O*-benzylhydroxylamine in ethanol. Elution with 7:3 (v/v) hexanes/EtOAc gave 0.51 g (65%) of **7e** as white crystals, mp 130–131 °C. 1H NMR (400 MHz, DMSO- d_6) δ 13.67 (s, 1H, OH), 7.91 (dd, 8.1, 1.6 Hz, 1H, H5), 7.69 (ddd, 8.4, 7.3, 1.6 Hz, 1H, H7), 7.40 (m, 7H, H6,8, Ph), 5.24 (s, 2H, OCH_2), 2.32 (s, 3H, CH_3). ^{13}C NMR (101 MHz, DMSO- d_6) δ 165.7, 160.2, 157.5, 152.5, 136.8, 133.6, 128.5, 128.3, 128.2, 124.3, 124.1, 116.2, 115.6, 98.7, 75.8, 15.0. IR (cm^{-1}) 3061 (w), 2918 (w), 2859 (w), 1704 (s), 1621 (m), 1549 (m), 1489 (m), 1452 (m). Anal. calcd for

$C_{18}H_{15}NO_4$: C, 69.89; H, 4.89; N, 4.53. Found: C, 69.88; H, 4.84; N, 4.72. UV/vis (MeOH) λ_{max} , nm (ϵ): 325 (1.11×10^4).

4-Hydroxy-3-(N-(2-phenylamino)ethanimidoyl)-2H-chromen-2-one (7f).—

Following method B, condensation of 0.31 g (1.5 mmol) of **4** with phenylhydrazine in ethanol gave 0.42 g (94%) of **7f** as yellow crystals, mp 224–225 °C (lit.^{28,75} 223–224, 236 °C). 1H NMR (400 MHz, DMSO- d_6) δ 15.67 (s, 1H, OH), 9.13 (s, 1H, NNHPh), 7.99 (ddd, 7.8, 1.7, 0.6 Hz, 1H, H5), 7.67 (ddd, 8.2, 7.3, 1.7 Hz, 1H, H7), 7.33 (m, 4H, H6,8, *m*-Ph), 6.95 (m, 3H, *o,p*-Ph), 2.74 (s, 3H, CH₃). ^{13}C NMR (101 MHz, DMSO- d_6) δ 177.9, 171.4, 161.3, 153.0, 145.2, 134.1, 129.5, 125.4, 123.8, 121.0, 119.3, 116.3, 113.0, 95.5, 17.0. IR (cm⁻¹) 3221 (m), 3118 (w), 3029 (w), 2954 (w), 1676 (s), 1605 (s), 1568 (m), 1465 (s), 1428 (m). MS m/z 218 (MH⁺). Anal. calcd for $C_{17}H_{14}N_2O_3$: C, 69.38; H, 4.79; N, 9.52. Found: C, 69.27; H, 5.02; N, 9.72.

4-Hydroxy-3-(N-(2-pyridylmethyl)ethanimidoyl)-2H-chromen-2-one (7g).—

Following method C, 0.32 g (1.6 mmol) of **4** was condensed with 2-(aminomethyl)pyridine in ethanol. Elution with 7:3 (v/v) hexanes/EtOAc gave 0.33 g (78%) of **7g** as white crystals, mp 158.5 °C. 1H NMR (400 MHz, DMSO- d_6) δ 8.65 (ddd, 4.9, 1.8, 0.9 Hz, 1H, 6-Py), 7.97 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.87 (td, 7.7, 1.8 Hz, 1H, 4-Py), 7.64 (ddd, 8.2, 7.2, 1.7 Hz, 1H, H7), 7.48 (m, 1H, 5-Py), 7.40 (ddd, 7.6, 4.9, 1.2 Hz, 1H, 3-Py), 7.28 (m, 2H, H6,8), 5.05 (d, 5.2 Hz, 2H, CH₂), 2.75 (s, 3H, CH₃). ^{13}C NMR (101 MHz, DMSO- d_6) δ 179.5, 176.2, 154.5, 153.0, 149.3, 137.3, 134.0, 125.9, 125.7, 123.6, 123.0, 122.1, 120.3, 116.2, 96.4, 48.7, 19.0. IR (cm⁻¹) 2360 (w), 1689 (s), 1606 (m), 1587 (m), 1567 (m), 1461 (s). MS m/z 295 (MH⁺). Anal. calcd for $C_{17}H_{14}N_2$: C, 69.38; H, 4.79; N, 9.52. Found: C, 69.38; H, 4.99; N, 9.73. UV/vis (DMSO) λ_{max} , nm (ϵ): 325 (2.04×10^4). UV/vis (MeOH) λ_{max} , nm (ϵ): 320 (1.60×10^4).

4-Hydroxy-3-(N-(3-pyridylmethyl)ethanimidoyl)-2H-chromen-2-one (7h).—

Following method C, 0.32 g (1.6 mmol) of **4** was condensed with 3-(aminomethyl)pyridine in ethanol. Elution with 7:3 (v/v) hexanes/EtOAc gave 0.43 g (75%) of **7h** as white crystals, mp 174 °C. 1H NMR (400 MHz, DMSO- d_6) δ 14.01 (s, 1H, NH), 8.67 (dd, 2.4, 0.8 Hz, 1H, 2-Py), 8.59 (dd, 4.8, 1.6 Hz, 1H, 6-Py), 7.93 (ddd, 7.8, 1.8, 0.5 Hz, 1H, H5), 7.86 (ddd, 7.9, 1.7, 0.9 Hz, 1H, 4-Py), 7.64 (ddd, 8.2, 7.2, 1.7 Hz, 1H, H7), 7.48 (ddd, 7.9, 4.8, 0.9 Hz, 1H, 5-Py), 7.28 (m, 2H, H6,8), 4.95 (d, 5.5 Hz, 2H, CH₂), 2.73 (s, 3H, CH₃). ^{13}C NMR (101 MHz, DMSO- d_6) δ 179.8, 176.6, 161.8, 153.0, 149.2, 149.1, 135.6, 134.1, 131.8, 125.8, 125.7, 124.0, 123.7, 120.0, 116.2, 45.0, 18.6. IR (cm⁻¹) 2981 (w), 2360 (w), 2341 (w), 1689 (s), 1593 (m), 1571 (m), 1464 (s). MS m/z 295 (MH⁺). Anal. calcd for $C_{17}H_{14}N_2O_3$: C, 69.38; H, 4.79; N, 9.52. Found: C, 69.07; H, 4.741; N, 9.63. UV/vis (DMSO) λ_{max} , nm (ϵ): 326 (2.02×10^4). UV/vis (MeOH) λ_{max} , nm (ϵ): 320 (1.87×10^4).

4-Hydroxy-3-(N-(4-pyridylmethyl)ethanimidoyl)-2H-chromen-2-one (7i).—

Following method C, 0.32 g (1.6 mmol) of **4** was condensed with 4-(aminomethyl)pyridine in ethanol. Elution with 7:3 (v/v) hexanes/EtOAc gave 0.40 g (70%) of **7i** as pink crystals, mp 151 °C. 1H NMR (400 MHz, DMSO- d_6) δ 14.03 (s, 1H, NH), 8.58 (m, 2H, 2,6-Py), 7.93 (m, 1H, H5), 7.62 (ddd, 8.3, 7.3, 1.7 Hz, 1H, H7), 7.35 (ddd, 4.4, 1.7, 0.8 Hz, 2H, 3,5-Py),

7.26 (m, 2H, H_{6,8}), 4.94 (d, 5.8 Hz, 2H, CH₂), 2.63 (s, 3H, NCCH₃). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 179.8, 177.2, 161.8, 153.1, 150.1, 145.2, 134.2, 125.8, 125.7, 123.7, 122.2, 120.1, 116.3, 46.2, 18.6. IR (cm⁻¹) 2923 (w), 2360 (w), 1686 (s), 1575 (s), 1484 (m), 1463 (m). MS *m/z* 295 (MH⁺). Anal. calcd for C₁₇H₁₄N₂O₃·0.25H₂O: C, 68.33; H, 4.89; N, 9.38. Found: C, 68.08; H, 4.62; N, 9.63. UV/vis (DMSO) λ_{max}, nm (ε): 326 (1.99 × 10⁴). UV/vis (MeOH) λ_{max}, nm (ε): 319 (1.83 × 10⁴).

4-Hydroxy-3-propionyl-2H-chromen-2-one (5a).—A solution of 2.0 g (9.8 mmol) of 3-hydroxycoumarin and 3.5 mL of POCl₃ in 10 mL of propionic acid was stirred and boiled under reflux for 45 min, then cooled to room temperature. The precipitate was collected by filtration and recrystallized from ethanol, giving 0.92 g (43%) of **5a** as white crystals, mp 123–124 °C (lit.^{76,77} 122–124, 130 °C). ¹H NMR (400 MHz, CDCl₃) δ 8.06 (m, 1H, H₅), 7.69 (ddd, 8.4, 7.3, 1.7 Hz, 1H, H₈), 7.33 (m, 2H, H_{6,7}), 3.24 (q, 7.1 Hz, 2H, CH₂), 1.22 (t, 7.2 Hz, 3H, CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 209.3, 178.5, 159.9, 154.8, 136.1, 125.7, 124.5, 117.1, 115.4, 101.0, 35.6, 8.0.

3-Butyryl-4-hydroxy-2H-chromen-2-one (5b).—A solution of 2.0 g (9.8 mmol) of 3-hydroxycoumarin and 3.5 mL of POCl₃ in 10 mL of butyric acid was stirred and boiled under reflux for 45 min, then cooled to room temperature. The precipitate was collected by filtration and recrystallized from ethanol, giving 1.6 g (69%) of **5b** as white crystals, mp 120–121 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.06 (ddd, 7.9, 1.7, 0.5 Hz, 1H, H₅), 7.69 (ddd, 8.4, 7.3, 1.7 Hz, 1H, H₈), 7.33 (m, 2H, H_{6,7}), 3.19 (t, 7.2 Hz, 2H, CH₂C=O), 1.76 (sext, 7.4 Hz, 2H, CH₂CH₃), 1.03 (t, 7.4 Hz, 3H, CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 208.8, 178.8, 159.9, 154.8, 136.1, 125.7, 124.5, 117.1, 115.5, 101.17, 43.7, 17.6, 13.9.

3-(N-Benzylpropanimidoyl)-4-hydroxy-2H-chromen-2-one (8a).—Following method B, condensation of 0.50 g (2.3 mmol) of **5a** with benzylamine in ethanol gave 0.65 g (91%) of **8a** as white crystals, mp 124–125 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.93 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H₅), 7.64 (ddd, 8.2, 7.3, 1.7 Hz, 1H, H₇), 7.44 (m, 4H, *o,m*-Ph), 7.38 (m, 1H, *p*-Ph), 7.28 (m, 2H, H_{6,8}), 4.93 (d, 5.6 Hz, 2H, NCH₂), 3.20 (q, 7.4 Hz, 2H, NCCH₂) 1.19 (t, 7.4 Hz, 3H, CH₃). IR (cm⁻¹) 2981 (w), 1690 (s), 1608 (m), 1566 (m), 1460 (m). Anal. calcd for C₁₉H₁₇NO: C, 74.25; H, 5.58; N, 4.66. Found: C, 74.15; H, 5.48; N, 4.66.

3-(N-Benzylbutanimidoyl)-4-hydroxy-2H-chromen-2-one (8b).—Following method B, condensation of 0.52 g (2.2 mmol) of **5b** with benzylamine in ethanol gave 0.66 g (93%) of **8b** as white crystals, mp 118–119 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.93 (ddd, 7.9, 1.8, 0.5 Hz, 1H, H₅), 7.63 (ddd, 8.2, 7.2, 1.7 Hz, 1H, H₇), 7.39 (m, 5H, Ph), 7.28 (m, 2H, H_{6,8}), 4.93 (d, 5.7 Hz, 2H, NCH₂), 3.15 (m, 2H, NCCH₂), 1.58 (m, 2H, CH₂CH₃), 1.03 (t, 7.3 Hz, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 180.2, 178.9, 161.4, 153.0, 136.2, 134.0, 128.9, 128.0, 127.7, 125.7, 123.6, 120.0, 116.1, 95.5, 47.1, 31.8, 20.3, 14.2. IR (cm⁻¹) 2968 (w), 1694 (m), 1607 (m), 1564 (s), 1465 (s). Anal. calcd for C₂₀H₁₉NO₃: C, 74.75; H, 5.96; N, 4.36. Found: C, 74.59; H, 5.81; N, 4.58.

3-Acetyl-4-hydroxy-6-nitro-2H-chromen-2-one (9).³²—Concentrated H₂SO₄ (20 mL) was cooled to 0 °C and 0.55 g (5.4 mmol) of KNO₃ was added, followed by 1.0 g (4.9 mmol) of **4**, which was added in portions. The resulting solution was stirred at 0 °C for 1 h and poured over 50 g of ice. The resulting precipitate was collected by filtration and washed with 100 mL of water, giving 1.10 g (90%) of **9** as a yellowish solid, mp 126–127 °C (lit.³² 230–231 °C). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.69 (d, 2.8 Hz, 1H, H5), 8.57 (dd, 9.1, 2.8 Hz, 1H, H7), 7.66 (d, 9.2 Hz, 1H, H8), 2.68 (s, 3H, CH₃).

4-Hydroxy-3-(N-pentylethanimidoyl)-6-nitro-2H-chromen-2-one (10a).²¹—By method B, condensation of 0.51 g (2.0 mmol) of **9** with pentylamine in ethanol gave 0.52 g (80%) of **10a** as yellowish crystals, mp 197–98 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 13.52 (s, 1H, NH), 8.62 (dd, 2.8, 1.2 Hz, 1H, H5), 8.42 (ddd, 9.0, 2.9, 0.6 Hz, 1H, H7), 7.49 (dd, 9.0, 0.8 Hz, 1H, H8), 3.64 (td, 7.0, 5.4 Hz, 2H, NCH₂), 2.66 (s, 3H, NCCH₃), 1.69 (quint, 7.2 Hz, 2H, NCH₂CH₂), 1.37 (m, 4H, (CH₂)₂CH₃), 0.91 (t, 7.2 Hz, 3H, CH₂CH₃). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 177.5, 176.2, 160.6, 156.7, 143.1, 128.3, 121.4, 120.3, 118.1, 95.8, 44.1, 28.3, 27.9, 21.9, 18.3, 13.8. Anal. calcd for C₁₈H₁₄N₂O₅: C, 60.37; H, 5.70; N, 8.80. Found: C, 60.48; H, 5.67; N, 8.85.

3-(N-Benzylethanimidoyl)-4-hydroxy-6-nitro-2H-chromen-2-one (10b).—Following method B, condensation of 0.52 g (2.1 mmol) of **9** with benzylamine in ethanol gave 0.59 g (84%) of **10b** as white crystals, mp 175 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 13.76 (s, 1H, NH), 8.64 (dd, 2.9, 0.4 Hz, 1H, H5), 8.44 (dd, 9.0, 2.9 Hz, 1H, H7), 7.52 (dd, 9.1, 0.4 Hz, 1H, H8), 7.42 (m, 5H, Ph), 4.94 (d, 4.9 Hz, 2H, CH₂), 2.73 (s, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 177.8, 176.6, 160.8, 156.8, 143.2, 135.7, 129.0, 128.5, 128.1, 127.8, 121.5, 120.4, 118.2, 96.25, 47.8, 18.7. IR (cm⁻¹) 3101 (w), 1717 (s), 1600 (m), 1567 (m), 1524 (m). Anal. calcd for C₁₈H₁₄N₂O₅: C, 63.90; H, 4.17; N, 8.28. Found: C, 63.84; H, 4.12; N, 8.37.

3-Acetyl-4-hydroxyquinolin-2(1H)-one (11).³⁵—A solution of 1.5 mL (12 mmol) of ethyl acetoacetate in 5 mL of xylene was stirred and heated under reflux as a solution of 1.5 mL (12 mmol) of methyl anthranilate in 4 mL of xylene was added dropwise. The resulting solution was stirred and boiled under reflux overnight, cooled to room temperature, then 10 mL of 10% (w/v) aq NaOH solution was added and the mixture was stirred for 1 h. The resulting precipitate was collected by filtration and washed with 20 mL of water, giving 1.07 g (45%) of **11** as a white solid, mp (dec) 300 °C (lit.^{35,78} 252–254, 248–250 °C). ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.40 (s, 1H, NH), 7.94 (dd, 7.9, 1.7 Hz, 1H, H5), 7.65 (t, 8.0, 1H, H7), 7.27 (d, 8.5, 1H, H8), 7.21 (t, 7.5, 1H, H6), 2.69 (s, 3H, CH₃).

Schiff bases **12a–i** were synthesized by the established procedures⁷⁹ and purified as follows, unless stated otherwise. Method D. A 0.04–0.07 M solution of 3-acetyl-4-hydroxy-2(1H)-quinolinone (**11**) and 1.1–1.2 equiv of a primary amine in the stated solvent was stirred and boiled under reflux from 4 to 16 h, then cooled to room temperature and concentrated to dryness by rotary evaporation. The product was purified by recrystallization from ethanol.

4-Hydroxy-3-(N-propylethanimidoyl)-2(1H)-quinolinone (12a).⁷⁹—Following method D, condensation of 0.37 g (1.8 mmol) of **11** with propylamine in ethanol gave 0.28 g

(63%) of **12a** as white crystals, mp 183–184 °C (lit.⁷⁹ 193–194 °C). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.56 (s, 1H, NH), 7.93 (dt, 7.9, 1.0 Hz, 1H, H5), 7.45 (ddd, 8.1, 7.1, 1.6 Hz, 1H, H7), 7.13 (ddd, 8.2, 1.2, 0.5 Hz, 1H, H8), 7.05 (ddd, 8.1, 7.1, 1.1 Hz, 1H, H6), 3.52 (td, 7.0, 5.3 Hz, 2H, NCH₂), 2.73 (s, 3H, NCCH₃), 1.68 (sext, 7.2 Hz, 2H, CH₂CH₃), 1.00 (t, 7.4 Hz, 3H, CH₂CH₃). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 179.9, 175.2, 164.7, 139.3, 132.5, 125.7, 120.7, 120.3, 114.9, 100.5, 44.8, 21.9, 17.8, 11.2. Anal. calcd for C₁₄H₁₆N₂O₂·0.5H₂O: C, 66.38; H, 6.77; N, 11.06. Found: C, 66.15; H, 6.32; N, 11.01.

4-Hydroxy-3-(N-isopropylethanimidoyl)-2(1H)-quinolinone (12b).⁷⁹—Following method D, condensation of 0.42 g (2.1 mmol) of **11** with isopropylamine in ethanol gave 0.26 g (52%) of **12b** as a white powder, mp 197–198 °C (lit.⁷⁹ 208–209 °C). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.55 (s, 1H, NH), 7.93 (dd, 8.0, 1.5 Hz, 1H, H5), 7.45 (ddd, 8.2, 7.1, 1.6 Hz, 1H, H7), 7.13 (dt, 7.9, 0.8 Hz, 1H, H8), 7.04 (ddd, 8.1, 7.1, 1.1 Hz, 1H, H6), 4.17 (m, 1H, CH), 2.75 (s, 3H, NCCH₃), 1.30 (d, 6.4 Hz, 6H, CH(CH₃)₂). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 179.9, 173.8, 164.7, 139.3, 132.5, 125.7, 120.7, 120.3, 114.9, 100.3, 45.0, 22.6, 17.9. Anal. calcd for C₁₄H₁₆N₂O₂·0.25H₂O: C, 67.59; H, 6.68; N, 11.26. Found: C, 67.32; H, 6.59; N, 11.14.

3-(N-Butylethanimidoyl)-4-hydroxy-2(1H)-quinolinone (12c).⁷⁹—Following method D, condensation of 0.43 g (2.1 mmol) of **11** with butylamine in ethanol gave 92 mg (17%) of **12c** as a white powder, mp 188–189 °C (lit.⁷⁹ 205–206 °C). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.55 (s, 1H, NH), 7.93 (m, 1H, H5), 7.45 (ddd, 8.2, 7.1, 1.6 Hz, 1H, H7), 7.12 (dt, 7.9, 1.0 Hz, 1H, H8), 7.04 (ddd, 8.1, 7.1, 1.1 Hz, 1H, H6), 3.55 (td, 7.1, 5.3 Hz, 2H, NCH₂), 2.73 (d, 1.7 Hz, 3H, NCCH₃), 1.65 (m, 2H, NCH₂CH₂), 1.44 (m, 2H, CH₂CH₃), 0.95 (t, 7.4 Hz, 2H, CH₂CH₃). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 179.9, 175.1, 139.3, 132.5, 125.7, 120.7, 120.3, 114.9, 100.5, 42.8, 30.5, 19.5, 17.8, 13.5. Anal. calcd for C₁₅H₁₈N₂O₂: C, 69.74; H, 7.02; N, 10.84. Found: C, 70.10; H, 6.99; N, 10.90.

4-Hydroxy-3-(N-isobutylethanimidoyl)-2(1H)-quinolinone (12d).—Following method D, condensation of 0.42 g (2.1 mmol) of **11** with isobutylamine in ethanol gave 0.42 g (78%) of **12d** as a white powder, mp 195–196 °C. ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.56 (s, 1H, NH), 7.94 (ddd, 7.8, 1.5, 0.7 Hz, 1H, H5), 7.46 (ddd, 8.2, 7.1, 1.6 Hz, 1H, H7), 7.12 (ddd, 8.2, 1.2, 0.5 Hz, 1H, H8), 7.05 (ddd, 8.1, 7.1, 1.1 Hz, 1H, H6), 3.41 (dd, 6.7, 5.4 Hz, 2H, CH₂), 2.73 (s, 3H, NCCH₃), 1.96 (m, 1H, CH), 1.01 (d, 6.7 Hz, 6H, CH(CH₃)₂). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 179.9, 175.3, 164.7, 139.3, 132.5, 125.7, 120.7, 120.3, 114.9, 100.5, 50.4, 27.8, 19.9, 17.9. Anal. calcd for C₁₅H₁₈N₂O₂: C, 69.74; H, 7.02; N, 10.84. Found: C, 70.08; H, 7.02; N, 10.92.

3-(N-sec-Butylethanimidoyl)-4-hydroxy-2(1H)-quinolinone (12e).⁷⁹—Following method D, condensation of 0.32 g (1.6 mmol) of **11** with *sec*-butylamine in ethanol gave 0.23 g (56%) of **12e** as a white powder, mp 167–168 °C (lit.⁷⁹ 177–178 °C). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.55 (s, 1H, NH), 7.93 (dd, 8.0, 1.5 Hz, 1H, H5), 7.45 (ddd, 8.5, 7.1, 1.6 Hz, 1H, H7), 7.13 (dt, 7.9, 0.9 Hz, 1H, H8), 7.04 (ddd, 8.1, 7.1, 1.1 Hz, 1H, H6), 4.01 (m, 1H, CH), 2.75 (s, 3H, NCCH₃), 1.64 (m, 2H, CH₂), 1.26 (d, 6.4 Hz, 3H, CHCH₃), 0.94 (t, 7.4 Hz, 3H, CH₂CH₃). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 180.0, 174.1, 164.7, 139.4,

132.5, 125.7, 120.7, 120.3, 114.9, 100.3, 50.3, 29.2, 20.2, 18.0, 9.9. Anal. calcd for $C_{15}H_{18}N_2O_2 \cdot 0.25H_2O$: C, 68.55; H, 7.10; N, 10.66. Found: C, 68.81; H, 4.76; N, 11.05.

4-Hydroxy-3-(N-pentylethanimidoyl)-2(1H)-quinolinone (12f).—Following method D, condensation of 0.26 g (1.3 mmol) of **11** with pentylamine in ethanol gave 34 mg (11%) of **12f** as a white powder, mp 196–197 °C. 1H NMR (500 MHz, $CDCl_3$) δ 8.83 (s, 1H, NH), 8.14 (d, 7.9 Hz, 1H, H5), 7.45 (ddd, 8.1, 7.2, 1.5 Hz, 1H, H7), 7.12 (m, 1H, H8), 6.97 (ddd, 8.1, 1.1, 0.5 Hz, 1H, H6), 3.53 (td, 7.2, 5.4 Hz, 2H, NCH_2), 2.83 (s, 3H, $NCCH_3$), 1.79 (quint, 7.3 Hz, 2H, NCH_2CH_2), 1.43 (m, 4H, $(CH_2)_2CH_3$), 0.95 (t, 7.2 Hz, 3H, CH_2CH_3). ^{13}C NMR (101 MHz, DMSO- d_6) δ 179.9, 175.2, 160.2, 139.3, 132.5, 131.9, 125.7, 120.8, 114.9, 100.5, 43.2, 28.5, 28.2, 21.7, 17.9, 13.9. Anal. calcd for $C_{15}H_{18}N_2O_2$: C, 69.74; H, 7.02; N, 10.84. Found: C, 69.86; H, 7.15; N, 10.51.

4-Hydroxy-3-(N-(2-hydroxyethyl)ethanimidoyl)-2(1H)-quinolinone (12g).—Following method D, condensation of 0.31 g (1.6 mmol) of **11** with ethanolamine in ethanol gave 0.27 g (70%) of **12g** as a white powder, mp 223–224 °C. 1H NMR (500 MHz, DMSO- d_6) δ 10.52 (s, 1H, NH), 7.93 (m, 1H, H5), 7.45 (ddd, 8.2, 7.1, 1.6 Hz, 1H, H7), 7.12 (ddd, 8.2, 1.1, 0.5 Hz, 1H, H8), 7.04 (ddd, 8.1, 7.1, 1.1 Hz, 1H, H6), 5.08 (s, 1H, OH), 3.62 (m, 2H, CH_2OH), 3.61 (q, 5.2 Hz, 2H, NCH_2), 2.74 (s, 3H, CH_3). ^{13}C NMR (101 MHz, DMSO- d_6) δ 179.8, 175.3, 164.8, 139.4, 132.4, 125.7, 120.7, 120.4, 114.8, 100.5, 59.2, 45.9, 18.0. Anal. calcd for $C_{13}H_{14}N_2O_3 \cdot 0.5H_2O$: C, 61.17; H, 5.92; N, 10.97. Found: C, 61.28; H, 6.11; N, 11.20.

3-(N-Cyclohexylmethylethanimidoyl)-4-hydroxy-2(1H)-quinolinone (12h).—Following method D, condensation of 0.33 g (1.61 mmol) of **11** with cyclohexylmethylamine in ethanol gave 0.35 g (72%) of **12h** as a white powder, mp 214–215 °C. 1H NMR (500 MHz, DMSO- d_6) δ 10.55 (s, 1H, NH), 7.94 (dd, 7.9, 1.6 Hz, 1H, H5), 7.45 (ddd, 8.1, 7.1, 1.6 Hz, 1H, H7), 7.12 (ddd, 8.1, 1.2, 0.5 Hz, 1H, H8), 7.04 (ddd, 8.1, 7.1, 1.1 Hz, 1H, H6), 3.42 (dd, 6.7, 5.4 Hz, 2H, NCH_2), 2.72 (s, 3H, CH_3), 1.71 (m, 5H, Cy), 1.16 (m, 6H, Cy). ^{13}C NMR (101 MHz, DMSO- d_6) δ 179.9, 175.2, 164.7, 139.3, 132.5, 125.7, 120.7, 120.3, 114.9, 100.6, 49.3, 37.1, 30.1, 25.8, 25.2, 17.9. Anal. calcd for $C_{18}H_{22}N_2O_2$: C, 72.46; H, 7.43; N, 9.39. Found: C, 72.28; H, 7.83; N, 9.63.

3-(N-Benzylethanimidoyl)-4-hydroxy-2(1H)-quinolinone (12i).⁷⁹—Following method D, condensation of 0.32 g (1.6 mmol) of **11** with benzylamine in ethanol gave 23 mg (5%) of **12i** as a white powder, 229–230 °C (lit.⁷⁹ 250–251 °C). 1H NMR (400 MHz, DMSO- d_6) δ 10.59 (s, 1H, NH), 7.91 (dd, 7.9, 1.5 Hz, 1H, H5), 7.41 (m, 6H, H7, Ph), 7.13 (ddd, 8.2, 1.1, 0.5 Hz, 1H, H8), 7.04 (ddd, 8.1, 7.1, 1.1 Hz, 1H, H6), 4.83 (d, 5.4 Hz, 2H, NCH_2), 2.79 (s, 3H, $NCCH_3$). Anal. calcd for $C_{18}H_{16}N_2O_3$: C, 73.95; H, 5.52; N, 9.58. Found: C, 73.68; H, 5.44; N, 9.49.

3-Acetyl-4-hydroxy-1-methyl-2(1H)-quinolinone (13).⁸⁰—A solution of 0.82 g (4.1 mmol) of **11**, 0.56 g (4.1 mmol) of K_2CO_3 , and 0.25 g (4.1 mmol) of MeI in 30 mL of dimethylformamide (DMF) was stirred at room temperature for 24 h, then poured onto ice. The resulting precipitate was collected by filtration and purified by flash chromatography on

silica eluting with 3:2 (v/v) hexanes/EtOAc, giving 0.23 g (25%) of **13** as white powder, mp 139–140 °C (lit.^{28,80,81} 137–138, 138, 140–142 °C). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.12 (m, 1H, H5), 7.82 (ddd, 8.7, 7.1, 1.6 Hz, 1H, H7), 7.55 (d, 8.7 Hz, 1H, H8), 7.34 (ddd, 8.0, 7.1, 0.9 Hz, 1H, H6), 3.57 (s, 3H, NCH₃), 2.73 (s, 3H, CH₃C=O).

3-(*N*-Benzylethanimidoyl)-4-hydroxy-1-methyl-2(1*H*)-quinolinone (**14**).⁸²—

Following method D, condensation of 0.21 g (0.96 mmol) of **13** with benzylamine in ethanol gave 0.25 g (86%) of **14** as white crystals, mp 163–164 °C (lit.^{83,84} 162–163, 159.5–161.5 °C). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.07 (ddd, 7.8, 1.7, 0.5 Hz, 1H, H5), 7.60 (ddd, 8.4, 7.1, 1.7 Hz, 1H, H7), 7.40 (m, 6H, H8, Ph), 7.16 (ddd, 8.0, 7.1, 1.0 Hz, 1H, H6), 4.85 (d, 5.4 Hz, 2H, CH₂), 3.47 (s, 3H, NCH₃), 2.77 (s, 3H, NCCH₃). MS *m/z* 307 (MH⁺). IR (cm⁻¹) 2938 (w), 1623 (s), 1605 (s), 1549 (m), 1451 (m). Anal. calcd for C₁₉H₁₈N₂O₂: C, 74.49; H, 5.92; N, 9.14. Found: C, 74.23; H, 6.07; N, 9.18.

Supplementary Material

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ABBREVIATIONS

BTS	<i>N</i> -benzyl- <i>p</i> -toluenesulfonamide
BHC	3-(<i>N</i> -butylethanimidoyl)ethyl)-4-hydroxy-2 <i>H</i> -chromen-2-one
EGTA	ethylene glycol bis(β-aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid
FAST	Fast Automated Spud Tracker
P_i	inorganic phosphate; MOPS, 3-morpholinopropane-1-sulfonic acid
S1	soluble head fragment of myosin

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Figure 1.

Myosin ATPase cycle. Myosin-ATP and -ADP-P_i (blue) bind weakly to actin and are shown in actin-detached states. All other myosin states (red) are shown in the actin-attached state along with their relative affinities for actin. The overall rate of attachment of myosin to actin, k_{att} , is limited by initial weak binding of the ADP-P_i state (K_w) followed by a transition to a strongly bound adenosine diphosphate (ADP) state coupled to P_i release (k_{ws}). A conformational change swings the lever arm domain resulting in the working step. After ADP release, ATP rebinds, which leads to weakly bound states. The image is modified from ref 4.

Figure 2.

Structures of blebbistatin (**1a**), *para*-aminoblebbistatin (**1b**), and 3-((*N*-butylethanimidoyl)ethyl)-4-hydroxy-2*H*-chromen-2-one (BHC) (**2**). The coumarin ring in **2** is inverted relative to the usual representation to show the proposed pharmacophore, consisting of the aromatic core, hydrogen bonding, and hydrophobic side chain.

Figure 3. Imine and enamine tautomers of 3-acyl-4-hydroxycoumarins ($X = O$) and 3-acyl-4-hydroxyquinolin-2-ones ($X = NR$).

Figure 4.

(A) Representative titrations of the analogues *versus* ATPase activity of skeletal myosin. BHC (2), red circles; **7i**, black squares; and **6b**, blue diamonds. The lines show the fits as described in the text. The error bars show the standard deviation for N determinations (see Table 1). (B) Linear aliphatic chain length of the BHC (2) analogues from Table 1 *versus* IC_{50} for skeletal (blue) and cardiac (red) myosin. The points are connected by the lines. Under these conditions, $K_{ATPase} = 8.7 \pm 1.5 \mu M$ and V_{max} ranges from 0.9 to $1.5 s^{-1} h^{-1}$. Conditions: $30^{\circ}C$, assay buffer with $32 \mu M$ actin, $2 mM$ ATP.

Figure 5. Effect of BHC (**2**) on *in vitro* actin-sliding velocity of skeletal myosin at 100 $\mu\text{g mL}^{-1}$. The error bars show the standard deviations and the red line shows the fit, giving $\text{IC}_{50} = 2.8 \pm 0.4 \mu\text{M}$ ($\pm$ is the standard deviation of the error of the fit). $T = 30^\circ\text{C}$.

Figure 6.

Interactions of **1a** and **7a** with skeletal S1 at 22–25 °C in assay buffer with 1 mM ATP. (A) Fluorescence of a mixture of **1a** (2 μM) and S1 was measured at 560 nm (λ_{ex} 440 nm). (B) Fluorescence of a mixture of **1a** (2 μM) and S1 (1 μM) was measured after incubating with **7a** for 1 h. Fluorescence without S1 (F_{-S1}) was subtracted from fluorescence with S1 (F_{+S1}) and normalized to maximal fluorescence (F_{max}). Curve fitting (red lines) is described in the Experimental Section. $N = 1$.

Figure 7.

Docking results. (A) Highest scoring pose of **1a** (green) and (B) highest scoring pose of **2** (yellow) docked to the cardiac myosin homology model (gray). The residues participating in ligand binding are shown as the light blue sticks and the polar interactions are shown as the red dashed lines. (C) Structure of *D. discoideum* myosin II (PDB: 1yv3, gray) showing **1a** (green) and ADP-VO₄ (red). (D) Residues (light blue) within 4 Å of the phenyl group of **7a** (orange) in the cardiac myosin model. Noncarbon atoms for ligands: red for oxygen, blue for nitrogen, and white for amide hydrogens.

Figure 8.

Representative effect of the BHC analogues on phrenic nerve stimulation-induced contraction of the mouse diaphragm. Muscle force represented by optical shortening of the diaphragm fibers in response to nerve stimulation at 1 Hz for 5 s (blue, vehicle; red, 100 μ M **7i**).

Scheme 1.

Scheme 2.

Scheme 3.

Product	Yield	R
12a (JB038)	63%	Pr
12b (JB039)	52%	<i>i</i> -Pr
12c (JB033)	17%	Bu
12d (JB040)	78%	<i>i</i> -Bu
12e (JB041)	56%	<i>sec</i> -Bu
12f (JB037)	11%	(CH ₂) ₄ Me
12g (JB047)	70%	(CH ₂) ₂ OH
12h (JB043)	72%	CH ₂ Cy
12i (JB036)	5%	Bn

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Table 1.

(A–G) Potencies (IC₅₀ Values) for Inhibition of Steady-State Actin-Activated Mg²⁺-ATPase Activity of Skeletal and Cardiac Myosin^a

Compound	Structure	IC ₅₀ ^a (μM)		IC ₅₀ ratio: cardiac/ skeletal	Compound	Structure	IC ₅₀ (μM)		IC ₅₀ ratio: cardiac/ skeletal
		(Number replicates, N)					(Number replicates, N)		
Table 1A: Bilibistatin									
1a		2.3 ± 0.3	0.5 ± 0.1	4.6	Table 1D: BHC analogues with benzene rings (continued)				
		(1)	(1)	(1)	7e		≥100	≥100	
1b		2.1 ± 0.7	0.5 ± 0.7	4.2	(JB063)		(1)	(1)	
		(1)	(1)	(1)	7f		27 ± 13	2.0 ± 1.0	13.5
Table 1B: BHC and analogues with linear aliphatic chains									
2		5.9 ± 1.2	2.1 ± 0.8	2.8	8a		≥100	≥100	
BHC		(3)	(3)		(JB064)		(1)	(1)	
6a		≥100	≥100		8b		≥100	≥100	
(JB032)		(1)	(1)		(JB066)		(1)	(1)	
6b		≥100	≥100		Table 1E: BHC analogues with pyridylmethyl groups				
(JB010)		(1)	(2)		7g		22 ± 11	6.8 ± 1.3	3.2
6c		100 ± 50	47 ± 9	2.1	(JB029)		(3)	(3)	
(JB009)		(3)	(3)		7h		17 ± 1	3.0 ± 0.9	5.6
6d		54 ± 14	31 ± 3	1.7	(JB030)		(3)	(3)	
(JB011)		(3)	(4)		7i		≥100	8.5 ± 0.8	≥12
					(JB031)		(3)	(3)	
Table 1F: BHC analogues with quinoline ring system									
6h		6.7 ± 0.8	2.0 ± 0.4	3.3	Table 1F: BHC analogues with quinoline ring system				
(JB005)		(3)	(3)		12a		≥100	≥100	
6i		2.9 ± 0.9	1.2 ± 1.0	2.4	(JB038)		(1)	(1)	
(JB052)		(3)	(3)		12b		≥100	≥100	
6j		92 ± 25	92 ± 52	1.0	(JB039)		(1)	(1)	
(JB006)		(1)	(2)		12c		≥100	≥100	
6k		37 ± 7	31 ± 4	1.2	(JB033)		(1)	(1)	
(JB007)		(3)	(3)		12d		≥100	≥100	
					(JB040)		(1)	(1)	
Table 1C: BHC analogues with branched-chain aliphatic groups									
6e		≥100	≥100		12e		≥100	≥100	
(JB003)		(3)	(2)		(JB041)		(1)	(1)	
6f		34 ± 4	≥100	≤0.34	12f		≥100	≥100	
(JB004)		(4)	(3)		(JB037)		(1)	(1)	
6g		≥100	≥100		12g		≥100	≥100	
(JB012)		(1)	(1)		(JB047)		(1)	(1)	
Table 1D: BHC analogues with benzene rings									
7a		7.3 ± 0.7	0.8 ± 0.2	9.1	12h		≥100	≥100	
(JB002)		(3)	(3)		(JB043)		(1)	(1)	
7b		≥100	≥100		12i		≥100	≥100	
(JB070)		(2)	(1)		(JB036)		(1)	(1)	
7c		4.9 ± 1.7	10 ± 4	0.5	14		≥100	≥100	
(JB069)		(3)	(3)		(JB075)		(1)	(1)	
7d		7.9 ± 2.5	0.8 ± 0.3	9.9	Table 1G: Nitrocarbazole BHC analogues				
(JB060)		(3)	(4)		10a		≥100	≥100	
					(JB096)		(1)	(1)	
					10b		≥100	≥100	
					(JB065)		(1)	(1)	

^aUncertainty (Δ) is the standard error of the fit in the combined data from N independent titration experiments (see text).

^aUncertainty (3) is the standard error of the fit to the combined data from N independent titration experiments (see text).

Uncertainty ($\pm$) is the standard error of the fit to the combined data from N independent titration experiments (see the text).

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Docking Results Listing the Contact Residues within 4 Å of the Highest Scoring Binding Pose of 1a and 2, with the Bolded Residues and Ligand Functional Groups Involved in Hydrogen Bonding

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BHC (2)		Blebbistatin (1a)	
Phe473	no interaction	D-ring	Phe466 Leu475

^aGene: MYH7; UniprotKB: Q9BE39 (MYH7_BOVIN).^bGene: MYH13; UniProtKB: Q9GJP9 (Q9GJP9_RABIT).

Table 3.

Summary of the Effects of the Compounds on Skeletal Muscle Contractility and Neurotransmission

compound	decrease in muscle shortening (%) at 100 μ M (N, number of replicates)	action potentials observed at paralyzing doses?	skeletal myosin ATPase IC50 < 100
Class 1 : Potency for Skeletal But Not Nerve Myosin			
2 (BHC)	96 $\pm$ 2 (5)	yes	yes
7i (JB031)	98 $\pm$ 3 (2)	yes	yes
Class 2: Potency for Both Nerve and Skeletal Myosin			
1a (blebbistatin)	99 (1)	no	yes
6j (JB006)	87 (1)	no	yes
6k (JB007)	72 $\pm$ 3 (2)	no ^a	yes
7g (JB029)	86 (1)	no	yes
Class 3: Higher Potency for Nerve versus Skeletal Myosin			
6a (JB032)	73 (1)	no ^a	no
6b (JB010)	80 (1)	no ^a	no
6e (JB003)	94 $\pm$ 7 (2)	no	no
12d (JB040)	71 (1)	no ^a	no
12e (JB041)	98 (1)	no	no
12f (JB037)	99 (1)	no	no
12g (JB047)	94 (1)	no	no
12h (JB043)	72 (1)	no ^a	no
Class 4: Poor Potency for Nerve and Skeletal Myosin			
6f (JB004)	22 $\pm$ 10 (2)	N.A.	no
6g (JB012)	21 (1)	N.A.	no
12b (JB039)	18 (1)	N.A.	no
12i (JB036)	16 (1)	N.A.	no
12a (JB038)	41 (1)	N.A.	no
12c (JB033)	48 (1)	N.A.	no

^aInhibitor concentrations >100 μ M were required to sufficiently paralyze the muscle to enable the action potential assessment. N.A. not applicable.