High-throughput screen, using time-resolved FRET, yields actin-binding compounds that modulate actin–myosin structure and function

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We have used a novel time-resolved FRET (TR-FRET) assay to detect small-molecule modulators of actin–myosin structure and function. Actin–myosin interactions play crucial roles in the generation of cellular force and movement. Numerous mutations and post-translational modifications of actin or myosin disrupt muscle function and cause life-threatening syndromes. Here, we used a FRET biosensor to identify modulators that bind to the actin–myosin interface and alter the structural dynamics of this complex. We attached a fluorescent donor to actin at Cys-374 and a nonfluorescent acceptor to a peptide containing the 12 N-terminal amino acids of the long isoform of skeletal muscle myosin’s essential light chain. The binding site on actin of this acceptor-labeled peptide (ANT) overlaps with that of myosin, as indicated by (a) a similar distance observed in the actin–ANT complex as in the actin–myosin complex and (b) a significant decrease in actin–ANT FRET upon binding myosin. A high-throughput FRET screen of a small-molecule library (NCC, 727 compounds), using a unique fluorescence lifetime readout with unprecedented speed and precision, showed that FRET is significantly affected by 10 compounds in the micromolar range. Most of these “hits” alter actin-activated myosin ATPase and affect the microsecond dynamics of actin detected by transient phosphorescence anisotropy. We conclude that the actin–ANT TR-FRET assay enables detection of pharmaceutically active compounds that affect actin structural dynamics and actomyosin function. This assay establishes feasibility for the discovery of allosteric modulators of the actin–myosin interaction, with the ultimate goal of developing therapies for muscle disorders.

The structural transition of the actin–myosin complex from the weak (lever arm up) to the strong (lever arm down) binding states during myosin’s ATPase cycle produces the force of muscle contraction (1). The molecular mechanism involves structural transitions at the interface between actin and myosin’s catalytic domain and within myosin’s light chain domain, which contains binding sites for essential (ELC)2 and regulatory light chains. Both light chains play important roles in regulating the actin–myosin interaction (2, 3). ELCs are highly conserved and are expressed as two isoforms, A1 and A2. The principal difference between them is that A1 contains an N-terminal extension (NTE) of 40–45 additional amino acids. Our previous work showed that when myosin binds to actin, the NTE of fast skeletal or cardiac ELC is in close proximity to actin (Fig. 1) (4, 5). N-terminal amino acids of NTE, specifically the first four amino acids (APKK), are highly conserved among skeletal and cardiac muscles and also across species (6). These positively charged lysine residues interact with the negatively charged C terminus glutamates of actin (7). The actin–NTE interaction is functionally relevant, because myosin isoforms having NTEs show higher catalytic efficiency and slower motility on actin (3, 8, 9).

Recent studies from our laboratory have explored the structural basis of ELC-mediated modulation of the actin–myosin interaction (4, 5, 10). Time-resolved FRET (TR-FRET), using a donor on actin and an acceptor on the A1 NTE of skeletal myosin subfragment 1 (S1), showed that the NTE plays an important role modulating myosin’s force-producing powerstroke (4). Similar studies with cardiac (ventricular) myosin S1 provided direct insight into the mechanism for perturbation of actin–myosin interactions by a cardiomyopathy mutation in the light chain domain (5). Many mutations or post-translational modifications in both actin and myosin cause life-threatening muscle disorders, and treatment options remain limited (11). We hypothesize that this TR-FRET approach can be used as a tool to screen for compounds that rescue defects in actomyosin structure and function. Small-molecule modulators of actomyosin structural dynamics represent potential leads for future drug development, and this search is greatly facilitated by recent developments in high-throughput FRET-based screening methods, which measure the effects of compounds

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2The abbreviations used are: ELC, essential light chain; NTE, N-terminal extension; TR-FRET, time-resolved FRET; S1, subfragment 1; FM, fluorescein 5-maleimide; HTS, high-throughput screening; FLTPR, fluorescence lifetime plate reader; TPA, transient phosphorescent anisotropy; ErI/A, erythrosine iodoacetamide; NCC, National Institutes of Health Clinical Collection.

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mole fraction of actin containing bound ANT (Equation 1 (see "Experimental procedures") to determine the interaction of the donor with multiple acceptors. Analysis change with [ANT], indicating that FRET was not affected by the presence of increasing concentrations of acceptor-labeled peptide (ANT) (Fig. 2, A). Use of this dabcyl-labeled nonfluorescent acceptor probe dabcyl to the N terminus of a large molecule library. Hits from this assay, defined as compounds (18) in the presence and absence of compounds from a small-molecule library. Here, we measured TR-FRET from actin to ANT with a high-precision fluorescence lifetime plate reader (FLTPR) (18) in the presence and absence of compounds from a small-molecule library. Hits from this assay, defined as compounds producing effects more than 4 S.D. from the mean, were analyzed further to determine their effects on actin-activated myosin ATPase activity, to evaluate the potential of this TR-FRET approach for drug discovery.

Results

Actin–ANT FRET biosensor

Time-resolved fluorescence decays of donor-labeled actin in the presence of increasing concentrations of acceptor-labeled peptide (ANT) (Fig. 2, A and B) were analyzed as indicated in Equation 1 (see "Experimental procedures") to determine the mole fraction of actin containing bound ANT (X_{DA}) (Fig. 2A). The fluorescence lifetime, \( t_{DA} \), of the bound complex did not change with [ANT], indicating that FRET was not affected by the interaction of the donor with multiple acceptors. Analysis revealed a \( K_d \) of 16.0 ± 1.2 \( \mu \)M (Fig. 2B), consistent with a previous report for the unlabeled peptide (17). This peptide had no effect on \( V_{max} \) and \( K_m \) of actin-activated ATPase of purified skeletal muscle acto-S1A1 (with NTE) (Fig. 2C) or the ATPase activity of rabbit skeletal myofibrils in activating or relaxing conditions (Fig. 2D).

The addition of ANT to FM actin decreased the donor lifetime (Fig. 3A, black to red), indicating FRET. Analysis of the FRET decay showed that the mean distance (R) between the two probes was 3.3 ± 0.2 nm, in very good agreement with the distance detected for probes at Cys-374 on actin and on Cys-16 at the NTE of A1 (4). The addition of NTE-containing S1s to the actin and ANT complex increased the lifetime (Fig. 3A, representative decay of S1s having NTE, red to blue), indicating that S1 either displaced or dissociated the actin-bound ANT. The analysis of FRET waveforms could not resolve the two possibilities. The FRET efficiency (\( E \)), calculated from the average lifetime in a model-independent analysis, is summarized in Fig. 3B. The addition of S1 isoforms with NTEs (skeletal S1, 75% of A1 + 25% of A2; skeletal S1A1, 100% A1; cardiac S1, 100% A1) had a significant effect on FRET, whereas the addition of an S1 isoform (S1A2, 0% A1) that does not contain an NTE had no effect on FRET between actin and ANT (Fig. 3B). These results indicate that ANT shares an overlapping binding site on actin with all tested ELC NTEs. The addition of ATP to a nucleotide-free mixture of actin, ANT, and S1 increased FRET between actin and ANT for the NTE-containing S1 isoforms, but not for S1A2 (Fig. 3B). This result serves as further evidence for overlapping ANT and NTE binding sites, as ATP causes dissociation of all S1 constructs from actin. The similarity of actin–ANT and actin–NTE interactions is further supported by the observation that both are sensitive to ionic strength; the addition of 0.1 M KCl to the actin–ANT complex also significantly decreased the rate of donor decay (Fig. 3A, red to green). Such sensitivity to the ionic strength specifies the electrostatic nature of this interaction and mirrors the model-based proposal that a positively charged NTE binds to a cluster of negatively charged residues in the C-terminal region of actin (19). These results clearly show that actin–ANT FRET is sensitive to the association of S1 NTEs with actin and also to ionic strength; both factors are known to be strong modulators of the actin–myosin interaction (19). Thus, we conclude that our actin–ANT FRET sensor has sufficient sensitivity for employment in a search for modulators of actin–ANT FRET in an HTS assay.

High-throughput screening of National Institutes of Health Clinical Collection (NCC) library to identify compounds that modulate actin–ANT FRET

Using the actin–ANT FRET sensor, we performed HTS of the NCC library (727 compounds). The NCC library is a collection of small molecules that have previously been tested in clinical trials and therefore have known safety profiles. The complete NCC library was applied to three 384-well black-wall/black-bottom Greiner plates with drug-free control (50 nl of DMSO) wells on each side of the individual plates (see “Experimental procedures”). For each screen, one set of drug plates was loaded with 2 \( \mu \)M FM-labeled actin (donor only), and the other set was loaded with 2 \( \mu \)M FM actin and 50 \( \mu \)M ANT (donor-acceptor). All plates were incubated for 20 min at 25 °C before reading. FM actin was excited with a 473-nm laser, and time-resolved fluorescence decay waveforms were read in the

High-throughput FRET assay detecting actin–myosin modulators

Figure 1. Model of actin–myosin complex with skeletal myosin S1 (heavy chain (blue), ELC (green), and regulatory light chain (red)) strongly bound to actin (gray) (19). Orange spheres show the labeling site on actin. The green circle indicates the location of the dabcyl-labeled N-terminal peptide (ANT).
FLTPR over a time course of 20 and 120 min. A two-exponential fit was used to obtain the lifetime of donor-only (H9270D) and donor-acceptor samples (H9270DA). Interfering (intrinsically fluorescent) compounds were removed using a control screen with 2 M unlabeled actin. A histogram of average FRET distribution from the NCC compounds was plotted and fitted to a Gaussian curve (Fig. 4A) to obtain a mean and S.D. FRET efficiency, \( E \), was computed on a well-to-well basis using Equation 1 (Fig. 4B). The \( Z' \) factor for this screen was calculated as 0.8 ± 0.1 using DMSO-only controls, which validates the robustness of this HTS screen (14, 20). This screen was performed in triplicate with three different preparations of donor and acceptor samples. Excellent agreement was observed in all three screens. Of 27, 21, and 23 hits across the three screens, a total of 10 compounds (Fig. 4B, red) reproducibly altered the average FRET by more than 4 S.D. greater than the mean of the control samples. This is a true hit rate of 35–45%. Compounds that did not reproducibly alter average FRET were considered false positives. The 10 remaining hit compounds were further tested in concentration–response FRET assays.

**FRET concentration–response assay**

Using the same condition as in the primary screen, we examined the dependence of FRET on the concentration of each reproducible hit compound (0.5–100 \( \mu \)M). Significant concentration-dependent effects on FRET were observed for most of the identified hits (Fig. 5). Actin–ANT FRET \( (E) \) for each compound was determined at each concentration and was subsequently normalized to the DMSO-only control \( (E_o) \). Concentration dependence of the normalized FRET change \( (E/E_o) \) was fitted using the Hill equation, which is routinely used in pharmacological studies to analyze concentration–response data of the drugs (21). All hit compounds decreased actin–ANT FRET at micromolar concentrations with notable differences in the apparent \( E_{50} \) (concentration needed for half-maximal effect) of the FRET curve (Fig. 5, A and B). \( E_{50} \) values are summarized in Table 1. Concentration dependence indicates that these compounds are interacting with actin. Concentration–response of two known myosin-binding drugs, OM and Myk 461, did not affect actin–ANT FRET (Fig. 5C). This control experiment further indicates the structural specificity of the identified hit compounds for actin. Future studies with compounds known to interact with actin will also be informative.

**Functional characterization of FRET hits on actomyosin ATPase activity**

Functional effects of the 10 hit compounds on actin-activated myosin ATPase (Fig. 6) were measured in a concentration-dependent manner. The concentration of actin (2 \( \mu \)M) and myosin was chosen to be the same as in the FRET measurements for consistency. None of the compounds altered Mg-ATPase of skeletal or cardiac S1 in the absence of actin (0.007 ± 0.002 S\(^{-1}\) in the absence of compound and 0.009 ± 0.002 S\(^{-1}\) in the presence of compounds). However, most of the Hit compounds affected the actin-activated ATPase of skeletal S1 (75% A1 and 25% A2) as well as cardiac S1 (100% A1) in a concentration-dependent manner. This is not surprising, because both skeletal and cardiac S1 contain predominantly the A1 isoform. Significant inhibition of actin-activated ATPase for both skeletal and cardiac myosin was observed for three compounds: fluorphenazine, thioradizine, and novantrone (Fig. 6). Honokiol activated both. Flutamide, dantrolene, and carvedilol had small and similar effects on both ATPases.
Some compounds showed significantly different effects on skeletal and cardiac myosin, suggesting the potential to identify isoform-specific effectors. Tegaserod had biphasic effects on cardiac acto-S1, and its inhibitory effects on skeletal acto-S1 were much more significant. Mefloquine activated cardiac acto-S1 at low concentrations but had no activating effect on skeletal acto-S1. Phenothiazine inhibited cardiac acto-S1, while slightly activating skeletal acto-S1.

Significant effectors show a good correlation of EC$_{50}$ values determined from FRET and ATPase assays (Table 1). Fluphenazine, thioradizine, and novantrone reduced actin-activated ATPase of both skeletal and cardiac S1 by 50% at their EC$_{50}$ values of 10–25 µM (Table 1). The decrease in ATPase activity induced by these three compounds was proportional to the decrease in FRET (Fig. 8, A and B), indicating that the functional effects are associated with structural changes in both species of the acto-S1 complex.

**Transient phosphorescent anisotropy**

Compound-induced changes in FRET (Fig. 5) suggested that these Hit compounds bind actin and modulate its structural properties. To test this possibility, we measured compound-induced changes in actin’s structural dynamics using transient phosphorescent anisotropy (TPA) of actin labeled at Cys-374 with erythrosine iodoacetamide (ErIA) (22–24). Two parameters of TPA decay characterize actin’s dynamics are (a) final anisotropy, related to the large-scale torsional flexibility of the filament (Fig. 7, A–D), and (b) lifetime, related to the probe environment near the C terminus of actin (Fig. 7, A, E, and F). Representative phosphorescence anisotropy decays in the presence of one of the Hit compounds, thioradizine (Fig. 7B), show concentration-dependent increase in the final anisotropy and decrease in signal intensity due to decrease in the phosphorescence lifetime. The effects of all hit compounds on both parameters of TPA decays are summarized in Fig. 7 (C–F). Most significant changes were observed in the presence of fluphenazine (Fig. 7, C and E), thioradizine, and novantrone (Fig. 7, D and F). The increase in actin anisotropy in the presence of fluphenazine and thioradizine suggests significant decrease in torsional flexibility of the filament. Novantrone-induced dramatic decrease in the anisotropy suggests large increase in torsional flexibility and/or fragmentation or depolymerization of actin filaments. Compounds that affected the anisotropy also decreased the erythrosine probe lifetime, suggesting that changes in actin’s flexibility are associated with changes in the probe environment, increasing exposure to the quenching effect of solvent oxygen. The changes in both anisotropy and phosphorescence lifetime were roughly proportional to changes in FRET efficiency (Fig. 8, C and D), suggesting that the compound-induced changes in FRET are associated with changes in actin structural dynamics. The compounds most effective in their effects on
TPAandFRETwerealsomosteffectivUNCHANGEdboundactiv-acti-
vatedmyosinATPase(Fig.6).Sevenothercompoundsinduced
also significant but less pronounced changes in TPA and in
actomyosin ATPase (Fig. 6), supporting previously observed
correlations between actin dynamics and the mechanism of
activation of myosin ATPase (10, 22, 23).

Discussion

Time-resolved FRET showed that a fluorescently labeled
12-amino acid peptide (ANT), corresponding to the NTE of A1
ELC (Fig. 1), binds to the C-terminal region of actin with
$K_d = 16 \mu M$. This actin–ANT interaction is affected by strong
binding of S1 isoforms and increasing ionic strength (Fig. 3),
suggesting overlap between ANT and myosin-binding regions
on actin. ANT itself did not alter actin-activated or myofibrillar
ATPase (Fig. 2, C and D). The lack of ANT effect on
$K_m$ has two possible explanations: (a) although ANT and S1
compete for actin binding in the absence of ATP (Fig. 3
B), the 16 $\mu M$ affinity of ANT (Fig. 2 B) for actin is about
2 orders of magnitude lower than that the submicromolar
affinity of S1 for actin (4), and (b) ANT and S1 do not
compete in the presence of ATP (Fig. 3 B).

The advantage of using low-affinity ANT to probe the actomy-
osin interface is that it is more likely to be susceptible to
compete with low-concentration pharmaceutically active com-
pounds, increasing the probability of detecting more “hit” com-
pounds. Therefore, FRET between probes on actin (donor) and
ANT (acceptor) was utilized as a sensor in a high-throughput
screening assay to identify compounds that alter the actin–
myosin interface. We identified 10 compounds from a small
(NCC) library that affected FRET (Figs. 4 and 5) in a concen-
tration-dependent manner. Most of them also altered the actin-
activated myosin ATPase of both skeletal and cardiac S1 as well
as the structural states of actin filament. Thus, we developed a
actin-based high-throughput assay where FRET changes are
associated with structural and functional changes in the whole
acto-S1 complex.

Validity of actin–ANT FRET sensor

The detected distance ($R = 3.3 \pm 0.2 \text{ nm}$) between probes on
actin and ANT in the actin–ANT complex is in good agreement
with the distance observed between Cys-16 of A1 ELC (skeletal,
2.9 ± 0.2 nm; cardiac, 3.5 ± 0.2 nm) and Cys-374 of actin in the
strongly bound acto-S1 complex (4, 5), indicating that ANT
binds to the A1 ELC-binding regions of actin. This possibility is
further supported by the effects of S1 binding on FRET signal
between actin and ANT (Fig. 3). In the absence of ATP, binding
of skeletal S1 (75% of A1 + 25% of A2), skeletal S1A1 (100% A1),
and cardiac S1 (100% A1) to the actin–ANT complex signifi-
cantly decreased FRET, whereas the addition of S1A2 (0% A1)
did not alter the FRET signal. Thus, only muscle S1s having
NTEs were capable of displacing the peptide from actin C-
terminal region. On the other hand, FRET was not affected in
the presence of ATP, where S1 binds weakly and is rotationally
disordered, and the distance between A1NTE and actin signif-
icantly increases compared with that in the strongly bound
complex (25, 26). FRET of actin–ANT complex was also signifi-
cantly decreased by an increase in ionic strength (0.1 M KCl),
further supporting the similarity between ANT–actin and
actin–A1NTE interaction; a structural model of the acto-S1A1
complex (19) shows that the positively charged N terminus of
A1 binds to a cluster of negatively charged residues in the C-
terminal region of actin (19). Thus, we conclude that our actin–

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\text{EC}_{50}$(FRET)</th>
<th>$\text{EC}_{50}$(ATPase) skeletal acto-S1</th>
<th>$\text{EC}_{50}$(ATPase) cardiac acto-S1</th>
</tr>
</thead>
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<tr>
<td>Honokiol</td>
<td>44 ± 2</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Tegaserod</td>
<td>29 ± 2</td>
<td>&gt;80</td>
<td>20.2 ± 5.1</td>
</tr>
<tr>
<td>Thiouracil</td>
<td>21 ± 3</td>
<td>25 ± 3</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>Flutamide</td>
<td>15 ± 2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Phenothiazine</td>
<td>7 ± 2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fluphenazine</td>
<td>25 ± 4</td>
<td>12.9 ± 3.9</td>
<td>11.0 ± 3.2</td>
</tr>
<tr>
<td>Dantrolene</td>
<td>48 ± 4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Novantrone</td>
<td>5.4 ± 0.7</td>
<td>11.3 ± 3.5</td>
<td>11.0 ± 2.0</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>48 ± 5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>21 ± 2</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not determined.

Figures 5. Concentration response of reproducible hit compounds on
FRET. A and B, hit compounds decreased FRET in a concentration-dependent
manner. C, myosin-specific compounds, OM and MYK461, did not show con-
centration dependence (control experiment). Error bars, S.D.
Figure 7. TPA of ErIA-labeled actin. A, schematic representation of actin’s microsecond dynamics detection by TPA measurements. \( I_v \) and \( I_h \) are vertically and horizontally polarized components of the emitted light used to calculate anisotropy (see “Experimental procedures”). B, TPA decays of ErIA-actin in the presence of increasing concentrations of thioridazine (0 – 100 \( \mu M \)), a representative compound.

C and D, final anisotropy in the presence of hit compounds, which decreases with the amplitude of reflecting global rotational dynamics of the actin filament. E and F, phosphorescence lifetime of decays measured in the presence of compounds, reflecting local structural change near the probe binding site. Error bars, S.D.

Figure 6. Concentration dependence of the ATPase activity of acto-S1. A and B, skeletal acto-S1. C and D, cardiac acto-S1. Data are normalized to the value of the ATPase in the absence of the compounds. Error bars, S.D.
ANT FRET sensor (a) mimics well actin–A1NTE binding, (b) has potential as a platform for the discovery of allosteric modulators of the actin–myosin interaction, and (c) can ultimately help in developing therapies to treat muscle disorders.

**Hit compounds identified in the HTS assay**

The principal goal of this HTS is to identify novel small-molecule effectors with therapeutic potential for disorders associated with mutations in actin or myosin. Small-molecule effectors designed to target and modulate striated and smooth muscle myosin isoforms for the treatment of disease are showing promise in preclinical and clinical trials (11, 27). In our work, we focus on modulation of actin. We identified 10 pharmacologically active compounds that affected both actin’s structure and functional interaction with myosin. The most pronounced effects were observed with fluphenazine, thioridazine, and novantrone. Fluphenazine and thioridazine are used as antipsychotic medicines (28), whereas Novantrone is used to treat multiple sclerosis (29) (Fig. 8). The effects of other identified compounds are less pronounced but still significant, particularly at higher concentrations. Tegaserod (30) and mefloquine (31) are used to treat conditions like irritable bowel syndrome and malaria. Dantrolene is a post-synaptic muscle relaxant and works on the ryanodine receptor (32). Carvedilol is already known as a β-blocker and is used to treat high blood pressure and heart failure (33). Honokiol, a traditional medicine, has several applications (34). Flutamide is an antagonist for androgen receptor and has reported anti-cancer activity (35).

All identified compounds are currently used as medications. Whereas these medicines are therapeutically effective, they also have significant side effects on muscle function, such as cardiac arrhythmia (29, 33). Thus, it is not surprising that these compounds were identified as hits, as they are truly related to the alteration of muscle function. Some of the undesirable side effects may be associated with the effects reported here on actin structure and interaction with myosin (Fig. 8). An effective drug must achieve a balance between therapeutic benefit and undesired side effects. This balance probably depends on the relative binding affinities to actin and other cellular targets. Our hit compounds affect actin when present at micromolar concentrations, indicating moderate actin binding affinity. If binding to therapeutically desirable targets is much stronger, therapeutic effects may be achieved at low enough doses to avoid effects on actin structure. On the other hand, drug-induced alteration of actin structure can be beneficial in cancer research as a method of inhibiting actin cytoskeleton function in malignant cells; for example, Novantrone is used in chemotherapy of certain cancers (31).

Our results have pharmacological implications, aiding understanding of the undesirable side effects of many medications. Actin is present in every human cell, and its interaction with multiple myosin isoforms and multiple actin-binding proteins is essential for cellular viability. Although actin is a very conserved protein, different actin isoforms cannot be substituted in living tissues (36, 37). Our HTS assay uses the muscle actin–myosin system for detection of compounds, but these compounds may also bind to cellular actin. Our future goals are to screen larger libraries in search of drugs that are specific for actin isoforms (i.e. skeletal muscle versus smooth muscle versus cardiac muscle versus nonmuscle), as desired for safe clinical
and sets the groundwork for the discovery of allosteric modulators. This tool can be used in the future to screen larger libraries of effectors that affect the functional interaction of actin and myosin.

**Conclusion**

Compound-specific structural and functional changes in actin–ANT FRET, phosphorescence, and actin-activated ATPase (Figs. 6 and 7) suggest that different hit compounds bind at different regions of actin. The binding and functional effects of each compound depend on the structure of the compound and the structure of the targeted region of actin. For example, two of the identified compounds, fluphenazine and thioridazine, are structurally similar to trifluoperazine, a known myosin ATPase inhibitor (38), and have very similar effects on the actomyosin ATPase and TPA decays (Figs. 6 and 7). A few compounds had different effects on skeletal and cardiac myosin (Fig. 6), suggesting interaction with myosin isoform-specific regions of the actomyosin interface. This interface is probably different for skeletal and cardiac S1, as indicated by significant differences in actin-activated ATPase.

We consider several possible mechanisms by which binding of hit compounds alters the interaction between the C terminus of actin and the NTE-binding site. Some compounds may bind to the C terminus of actin and alter its local structure. Other compounds may bind to other regions of actin and have allosteric effects. Other compounds may alter the microsecond flexibility of actin (22–24). Each mode of binding could result in displacement of ANT, as indicated by the decrease in FRET (Fig. 5), decrease in phosphorescence lifetime (Fig. 7) due to increased exposure of the probe to buffer oxygen, and/or changes in flexibility of actin due to local and allosteric changes in the monomers. Examples of long-range allosteric changes in actin were previously documented using site-specific modifications (39).

Our previous studies on actin-binding proteins demonstrated coupling between changes in actin flexibility (measured by TPA) and functional interactions with myosin and other actin-binding proteins (22–24). This kind of coupling is also observed in the present studies (Fig. 8).

None of these compounds has any effect on basal Mg-ATPase (without actin) of either skeletal or cardiac S1, confirming their actin specificity. In addition, FRET concentration–response with two known myosin-specific compounds (11, 27) showed no effects (Fig. 5), providing further indication that compounds identified in this screen act on actin to affect actomyosin function. This is unique, because previous drug discovery campaigns in this field used myosin as the primary target (11, 27). Our future screening of larger libraries will focus on finding compounds that are specific for skeletal or cardiac muscle.

**Mechanism of action**

Using the high precision of our TR-FRET technology, coupled with structure-based design of an intermolecular FRET biosensor involving actin and ANT, we have established and validated an HTS platform that can detect small-molecule effectors that affect the functional interaction of actin and myosin. This tool can be used in the future to screen larger libraries and sets the groundwork for the discovery of allosteric modulators of other actin-binding proteins of interest where protein–protein interactions are targeted.

**Experimental procedures**

**Protein preparations and labeling**

Actin was prepared from rabbit skeletal muscle by extracting acetone powder in cold water, as described previously (23). Cys-374 in F-actin was labeled with the FRET donor/phosphorescent probe as follows. FM/ErlA (Life Technologies, Inc.), freshly dissolved in N,N-dimethylformamide (500 μM), was mixed with 50 μM F-actin and incubated for 1 h at 25 °C, followed by 18 h at 4 °C. Labeling was terminated by adding 10 mM DTT. After 30 min of sedimentation at 350,000 × g, the F-actin pellet was suspended in G-Mg buffer (5 mM Tris, 0.5 mM ATP, 0.2 mM MgCl₂, pH 7.5) followed by clarification at 300,000 × g for 10 min. Actin was again polymerized for 30 min at 25 °C in the presence of 3 mM MgCl₂ and centrifuged at 300,000 × g for 10 min, and the pellet was suspended in F-Mg buffer (3 mM MgCl₂, 10 mM Tris, pH 7.5) containing 0.2 mM ATP. The labeled F-actin was immediately stabilized against depolymerization and denaturation by adding 1 molar eq of phalloidin.

Skeletal muscle S1 was obtained by α-chymotryptic digestion of rabbit skeletal muscle myosin (10), and β-cardiac S1 was prepared by α-chymotryptic digestion of bovine β-cardiac myosin (5) as described previously. Isoforms of skeletal muscle S1, S1A1 and S1A2, were purified as reported in our earlier study (10).

**The N-terminal peptide**

The N-terminal peptide of rabbit skeletal A1 ELC was synthesized and purified by the LifeTein Co. Unlabeled peptide was acetylated at the N terminus and amidated at the C terminus: Ac-APKKDVKKPVAA-NH₂ (Mᵣ 1292.6). The labeled peptide had dabcyl conjugated to the first A at the N terminus, dabcyl-APKKDVKKPVAA-NH₂ (Fig. 1), and amidated C terminus (Mᵣ 1501.85). The synthesized peptides were purified and converted to HCl salts to remove residual salts from the synthesis procedure and were provided as lyophilized powder. The peptide was >99% pure, as determined by HPLC and MS. Before experiments, peptides were dissolved in H₂O (added directly to the vial), aliquoted, and stored at −20 °C. The final concentration of unlabeled peptide was determined using the provided molecular weight, and the concentration of dabcyl-labeled peptide (ANT) was calculated by measuring absorbance at 434 nm, using the molar extinction coefficient of dabcyl, 22,850 cm⁻¹ M⁻¹.

**Fluorescence data acquisition**

Fluorescence lifetime measurements were carried out by a high-precision FLTPR (Fluorescence Innovations, Inc., Minneapolis, MN) (13–15). FM-labeled donor actin was excited with a 473-nm microchip laser (Bright Solutions, Cura Carpignano, Italy), and emission was filtered with 488-nm long pass and 517/20-nm band pass filters (Semrock, Rochester, NY) (13, 18). This instrument enables high-throughput fluorescence lifetime detection at high precision by utilizing a unique direct waveform-recording technology (40). The performance of this
High-throughput FRET assay detecting actin–myosin modulators

FLTPR has been previously demonstrated with FRET-based HTS that targets sarcoplasmic reticulum Ca-ATPase (SERCA) and the ryanodine receptor (14, 15).

**Pilot screen with NCC library**

The NCC compounds (727 compounds) were received in 96-well plates and were reformatted into 384-well polypropylene intermediate plates (Greiner Bio-One, Kremsmunster, Austria) and stored at 20°C. Before screening, 2 pound plates were equilibrated to room temperature (25°C). Santa Clara, CA) and stored at 20°C. Before screening, 2 pound plates were equilibrated to room temperature (25°C). Beforescreening,compoundswerepreparedby transferring 50 nl of the 10 mM compound stocks or DMSO from the source plates to 384-well black polypropylene plates (Greiner), using an Echo 550 acoustic dispenser (Labcyte). NCC compounds were formatted in three plates, with the first two and last two columns loaded with 50 nl of DMSO and used for drug-free controls. Final concentration of the compounds was 10 μM. These assay plates were then heat-sealed using a PlateLoc Thermal Microplate Sealer (Agilent Technologies, Santa Clara, CA) and stored at −20 °C. Before screening, compound plates were equilibrated to room temperature (25°C). Before screening, 2 μM FM (donor)-labeled actin without or with 50 μM acceptor labeled (ANT) peptide (50 μl/well) was dispensed by a Multidrop Combi Reagent Dispenser (Thermo Fisher Scientific) into the 384-well assay plates containing the compounds. Plates were incubated at room temperature for 20 min before recording data with the FLTPR.

**TR-FRET and HTS data analysis**

TR-FRET waveforms were analyzed globally by model-independent and -dependent methods as described previously (4, 5). The observed donor-only (FM actin) waveform \( F_{D\text{obs}}(t) \) was fitted by a simulation \( F_{D\text{sim}}(t) \), consisting of a multieponential decay \( F_{D}(t) \) (Fig. 3A, black) convolved with the instrument response function IRF(t). The observed donor + acceptor waveform (FM actin + ANT) \( F_{D+A\text{obs}}(t) \) (Fig. 3A, red, blue, magenta, and green) was fitted by a multieponential function using the same approach. The model-independent ensemble-average FRET efficiency \( E \), which is equivalent to the result of a steady-state fluorescence measurement, is given by the equation,

\[
E = 1 - \frac{\langle \tau_{DA} \rangle}{(\langle \tau_D \rangle)}
\]

where \( \tau_{DA} \) is the time-resolved fluorescence waveform of the donor-acceptor complex, \( \langle \tau_D \rangle \) is the fraction of actin-attached donors that are bound to and transferring energy to acceptor-labeled ANT, \( R \) is the effective donor-acceptor distance in the complex, and \( R_0 \) is the distance (3.6 nm) at which FRET is 50% for this donor–acceptor pair (4).

Waveforms for each well in HTS were fitted by a two-exponential decay function using least-squares minimization. The FRET efficiency \( E \) was determined as the fractional decrease in donor fluorescence lifetime due to the acceptor, \( E = 1 - \frac{\langle \tau_{DA} \rangle}{(\langle \tau_D \rangle)} \).

Assay quality was determined based on controls (DMSO-only samples) on each plate, as indexed by the Z’ factor, a value of 0.5 or higher indicating excellent assay quality (14, 20). A compound was considered a hit if it changed \( E \) by >4 S.D. relative to that of control samples (\( E_0 \)) that were exposed to 0.1% DMSO. The threshold may be further adjusted to constrain the number of hits according to the resources available for evaluation via secondary (orthogonal) assays.

**FRET concentration–response assay**

The hit compounds were dissolved in DMSO to make a 10 mM stock solution, which was serially diluted in 96-well mother plates. Hits were screened at eight concentrations (0.5–100 μM). Compounds (1 μl) were transferred from the mother plates into 384-well assay plates using a Mosquito HV liquid handler (TTP Labtech Ltd., Hertfordshire, UK). The same procedure of dispensing as for the pilot screening was applied in the FRET concentration–response assays. Concentration dependence of the FRET change was fitted using the Hill equation (21),

\[
E = E_0 + \left( \frac{E_{\text{max}}C}{EC_{50} + C} \right)
\]

where \( E_0 \) are FRET in the presence and in the absence of the compound, \( E_{\text{max}} \) is the maximum effect, \( C \) is the drug concentration, \( EC_{50} \) is the drug concentration for which 50% of maximum effect is obtained, and \( \alpha \) is the Hill coefficient of sigmoidicity. Concentration–response assays of two known myosin-specific compounds, OM and Myk 461, were also done as controls to validate the structural specificity of the compounds.

**Actin-activated ATPase**

Actin-activated S1 ATPase activity was measured at 25 °C in F-Mg buffer containing 3 mM ATP with 2 μM F-actin and 5 μM skeletal/cardiac S1. Concentration–response of drugs (0–100 μM) on ATPase was measured in the presence of the compounds, and the \( EC_{50} \) was determined by fitting the data to a Hill plot. The ATPase concentration–response data were plotted as normalized with reference to the ATPase of actomyosin in the absence of compounds (Fig. 6). Basal Mg-ATPase of S1 was measured under the same conditions in the absence of actin.

**Transient phosphorescent anisotropy**

Phalloidin-stabilized ErIA–F-actin was diluted in F-Mg buffer to 2.0 μM, and compounds were added at specified concentrations (0–100 μM). To maximize phosphorescence signals and prevent photobleaching of the dye, oxygen was removed from the sample by a 5-min incubation with glucose oxidase (55 μg/ml), catalase (36 μg/ml), and glucose (45 μg/ml). Phosphorescence was measured at 25 °C as described previously (10). ErIA actin was excited with a vertically polarized 1.2-ns pulse from an FDSS 532-150 laser (CryLas) at 532 nm, operating at a repetition rate of 100 Hz. Phosphorescence emission was

\[
\langle \tau_{DA} \rangle/(\langle \tau_D \rangle) = 1 + R/R_0^{-6}
\]
selected by a 670-nm glass cut-off filter (Corion), detected by a photomultiplier (R928, Hamamatsu), and digitized by a transient digitizer (CompuScope 14100, GaGe) at a time resolution of 1 μs/channel. The TPA decay was calculated as \( r(t) = (I_s(t) - G I_m(t))/I_s(t) + 2GI_m(t) \), where \( I_s(t) \) and \( I_m(t) \) are vertically and horizontally polarized components of the emission signal, detected at 90° with a single detector and a polaroid sheet polarizer that alternated between the two orientations every 500 laser pulses. \( G \) is an instrumental correction factor, determined by performing the measurement with horizontally polarized excitation, for which the corrected anisotropy value is set to zero. TPA decays were obtained by recording 10 cycles of 1000 pulses (500 in each orientation of the polarizer), corresponding to a total acquisition time of about 2 min. TPA decays were analyzed by calculating the final anisotropy \( r_\infty \), defined as the average value of \( r \) in the time window from 400 to 500 μs, which has been shown previously to provide the most sensitive and precise measurement of actin’s microsecond rotational dynamics (10). The phosphorescence intensity and mean lifetime were calculated as described previously (41).

**Author contributions**—P. G., E. P., and D. D. T. designed the research. P. G. and E. P. prepared samples, performed biochemical experiments, and analyzed the data. K. C. P. designed, constructed, and maintained the FLTPR. P. G., E. P., and B. D. G. acquired and analyzed fluorescence data. P. G., E. P., and D. D. T. wrote the paper.

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