

Piyali Guhathakurta¹, Ewa Prochniewicz¹, Benjamin D. Grant², Kurt C. Peterson², and David D. Thomas^{1,3,*}

From the ¹Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota; ²Fluorescence Innovations Inc., Minneapolis, Minnesota; ³Photonic Pharma LLC, Minneapolis, Minnesota

Running Title: *High-throughput FRET assay detecting actin-myosin modulators*

*To whom correspondence should be addressed: David D Thomas, Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis; e-mail: ddt@umn.edu. Tel: (612) 625-0957.

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ABSTRACT

We have used a novel time-resolved fluorescence resonance energy transfer (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 C374, and a non-fluorescent 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 pharmacologically 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.

Introduction

The structural transition of the actin-myosin complex from the weak (*W*, lever arm up) to the strong (*S*, 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 (CD), and within myosin's light chain domain (LCD), which contains binding sites for essential (ELC) and regulatory light chains (RLC). 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 to 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, since myosin isoforms having NTE's show higher catalytic efficiency and slower motility on actin (3,8,9).

Recent studies from our lab have explored the structural basis of ELC-mediated modulation of the actin-myosin interaction (4,5,10). Time-resolved fluorescence resonance energy transfer (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 LCD (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 on the distance between donor and acceptor probes on interacting proteins (1,12-15).

In the present study, we labeled actin at C374 with a fluorescent donor, fluorescein-5-maleimide (FM), and attached the non-fluorescent acceptor probe dabcyI to the N-terminus of a 12-aa peptide derived from the amino terminus of NTE of rabbit skeletal muscle A1 (Fig. 1). Use of this dabcyI-labeled peptide, designated ANT, was inspired by previous reports showing that the first 13 amino acid residues of NTE are important regulators of the actin-myosin interaction (16) and affect contractility of muscle cells (17). A key advantage of ANT, over our previously used acceptor-labeled myosin (4), is that it can be synthesized and purified in large quantity, thus facilitating large-scale high-throughput

screening (HTS). We hypothesized that compounds affecting the actin-ANT interaction are likely to perturb structural and enzymatic properties of actin-myosin. 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 standard deviations (SD) 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. 2A, B) were analyzed as indicated in $FD+Aobs(t) = (1 - X_{DA})FD(t) + X_{DA}FDA(t)$, Eq. (1), to determine the mole fraction of actin containing bound ANT (X_{DA}), Fig. 2A). The fluorescence lifetime τ_{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 $16.0 \pm 1.2 \mu M$ (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).

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 C374 on actin and on C16 at the NTE of A1 (4). 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 $\langle E \rangle$, calculated from the average lifetime in a model-independent analysis, is

summarized in (Fig. 3B). Addition of S1 isoforms with NTE's (skeletal S1: 75% of A1 + 25% of A2, skeletal S1A1: 100% A1 and cardiac S1:100 % A1) had a significant effect on FRET, whereas 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 NTE's. 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: addition of 0.1M 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 NTE's 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 a HTS assay.

High-throughput screening of 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 procedure). For each screen, one set of drug plates was loaded with 2 μ M FM labeled actin

(donor only) and the other set was loaded with 2 μ M FM actin and 50 μ M ANT (donor-acceptor). All plates were incubated 20 minutes at 25°C prior reading. FM actin was excited with a 473 nm laser and TR-fluorescence decay waveforms were read in the FLT-PR over a time course of 20 and 120 min. A two-exponential fit was used to obtain the lifetime of donor-only (τ_D) and donor-acceptor samples (τ_{DA}). Interfering (intrinsically fluorescent) compounds were removed using a control screen with 2 μ 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 SD. FRET efficiency $\langle E \rangle$ 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. Out of 27, 21, and 23 Hits across the three screens, a total of ten compounds (Fig. 4B, red) reproducibly altered the average FRET by more than 4SD 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 ten 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 μ 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_0). Concentration dependence of the normalized FRET change (E/E_0) 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 EC_{50} (concentration needed for half-maximal effect) of the FRET curve (Fig. 5A, B). EC_{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 ten Hit compounds on actin-activated myosin ATPase (Fig. 6) were measured in a concentration-dependent manner. The concentration of actin (2 μ 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, since 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: fluphenazine, thioradazine and novantrone (Fig. 6). Honokiol activated both. Flutamide, Dantrolene and carvediol 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, thioradazine and novantrone reduced actin-activated ATPase of both skeletal and cardiac S1 by 50% at their EC_{50} values of 10-25 μ M (Table 1). The decrease in ATPase activity induced by these three compounds was proportional to the decrease in FRET (Fig. 8A 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 Cys374 with erythrosine iodoacetamide (EriA) (22-24). Two parameters of TPA decay characterize actin's dynamics: (1) final anisotropy, related to the large-scale torsional flexibility of the filament (Fig. 7A-D), and (2) lifetime, related to the probe environment near the C-terminus of actin (Fig. 7A,E,F). Representative phosphorescence anisotropy decays in the presence of one of the Hit compounds thioridazine (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,E) thioridazine and novantrone (Fig. 7 D,F). The increase in actin anisotropy in the presence of fluphenazine and thioridazine 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 which 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. 8C, 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 TPA and FRET, were also most effective in changing actin-activated myosin ATPase (Fig. 6). Other 7 compounds induced 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 $K_d = 16 \mu\text{M}$. 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. 2C, D). The lack of ANT effect on K_m has two possible explanations: (1) Although ANT and S1 compete for actin binding in the absence of ATP (Fig. 3B), the $16 \mu\text{M}$ affinity of ANT (Fig. 2B) for actin is about two orders of magnitude lower than that the submicromolar affinity of S1 for actin (4) and (2). ANT and S1 does not compete in the presence of ATP (Fig. 3B). The advantage of using low-affinity ANT to probe the actomyosin interface is that it is more likely to be susceptible to compete with low-concentration pharmaceutically active compounds, increase the probability to detect more "Hit" compounds. 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 ten compounds from a small (NCC) library that affected FRET (Fig. 4,

Fig. 5) in a concentration-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 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 C16 of A1 ELC (skeletal, 2.9 ± 0.2 and cardiac $3.5 \pm 0.2 \text{ nm}$) and C374 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 significantly decreased FRET, whereas addition of S1A2 (0% A1) did not alter the FRET signal. Thus, only muscle S1s having NTE's 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 significantly increases in comparison to that in the strongly bound complex (25,26). FRET of actin-ANT complex was also significantly decreased by increase in ionic strength (0.1M KCl), further supporting similarity of interaction between ANT and A1NTE with actin: structural model of 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 (i) our actin-ANT FRET sensor mimics well actin-A1NTE binding (ii) has potential as a platform for the discovery of allosteric modulators of the actin-myosin interaction (iii) 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 ten pharmacologically active compounds that affected both actin's structure and functional interaction with myosin. Most pronounced effects were observed with fluphenazine, thioridazine and novantrone. Fluphenazine and thioridazine are used as antipsychotic medicines (28), while novantrone is used to treat multiple sclerosis (29). (Fig. 8). The effects of other identified compounds are less pronounced, but still have significant effects, particularly at higher concentrations. Tegaserod [30] (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 BP 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. While 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 μM 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, Novatrone 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 for drugs that are specific for actin isoforms, i.e., skeletal muscle vs smooth muscle vs cardiac muscle vs nonmuscle, as desired for safe clinical applications. Subsequent efforts in medicinal chemistry will be needed to enhance specificity and reduce side effects.

Mechanism of action

Compound-specific structural and functional changes in actin-ANT FRET, phosphorescence and actin-activated ATPase (Fig. 6, Fig. 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 TFP (trifluoperazine), a known myosin ATPase inhibitor (38), and have very similar effects on the actomyosin ATPase and TPA decays (Fig. 6, Fig. 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, confirm 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, since 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.

Conclusion

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 a 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). C374 in F-actin was labeled with the FRET donor/phosphorescent probe as follows: fluorescein-5-maleimide (FM)/erythrosin-iodoacetamide (ErIA) [Life technologies, OR, USA], freshly dissolved in DMF (500 μ M), was mixed with 50 μ M F-actin and incubated for 1 hour at 25°C followed by 18 h at 4°C. Labeling was terminated by adding 10 mM DTT. After 30 min sedimentation at 350,000xg, 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 x g for 10 minutes. Actin was again polymerized for 30 min at 25°C in the presence of 3 mM MgCl₂, centrifuged at 300,000xg 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 equivalent 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 Company (NJ, USA). Unlabeled peptide was acetylated at the N-terminus and amidated at the C-terminus: Ac-APKKDVKKPVAA-NH₂ (MW 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 (MW 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 mass spectrometry. 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, 22850 cm⁻¹M⁻¹.

Fluorescence data acquisition

Fluorescence lifetime measurements were carried out by a high-precision fluorescence lifetime plate reader (FLTPR, Fluorescence Innovations, Inc., Minneapolis, MN) (13-15). FM labeled donor actin was excited with a 473 nm microchip laser (Bright Solutions, 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 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 NIH clinical collection 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) using a multichannel liquid handler, BioMek FX (Beckman Coulter, Miami, FL). Assay plates were prepared by 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. Prior to screening, compound plates were equilibrated to room temperature (25°C). Prior to screening, 2 μM FM (donor) labeled actin without or with 50 μM acceptor labeled (ANT) peptide (50 μL/well) were 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 in model-independent and dependent methods as described previously (4,5). The observed donor-only (FM actin) waveform $F_{Dobs}(t)$ was fitted by a simulation $F_{Dsim}(t)$, consisting of a multiexponential decay $F_D(t)$ (Fig 3A, black) convolved with the instrument response function $IRF(t)$. The observed donor + acceptor waveform (FM actin+ANT) $F_{D+Aobs}(t)$ (Fig 3A, red, blue, magenta and yellow) was fitted by a multiexponential function using the same approach. The model-independent ensemble-average FRET efficiency $\langle E \rangle$, which is equivalent to the result of a steady-state fluorescence measurement is given by $\langle E \rangle = 1 - \langle \tau_{DA} \rangle / \langle \tau_D \rangle$, where brackets indicate average. The model-dependent structural state was resolved using

$$F_{D+Aobs}(t) = (1 - X_{DA})F_D(t) + X_{DA}F_{DA}(t), \quad \text{Eq. (1)}$$

$$F_D(t) = \exp(-t/\langle \tau_D \rangle), \quad F_{DA}(t) = \exp(-t/\langle \tau_{DA} \rangle), \\ \langle \tau_{DA} \rangle / \langle \tau_D \rangle = 1 + (R/R_0)^{-6},$$

where $F_{DA}(t)$ is the TR-fluorescence waveform of the donor-acceptor complex, X_{DA} 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: $E = 1 - \langle \tau_{DA} \rangle / \langle \tau_{DA} \rangle$.

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 SD 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 μ M). Compounds (1 μ L) 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)

$$E = E_0 + (E_{\max} C^\alpha / EC_{50}^\alpha + C^\alpha)$$

where E and E_0 are FRET in the presence and in the absence of the compound, $E_{\max}$ is the maximum effect, C is the drug concentration, EC_{50} is the drug concentration for which 50% of maximum effect is obtained and α is the Hill coefficient of sigmoidicity. Concentration–response 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 μ M F-actin and 5 μ M of skeletal/cardiac S1. Concentration–response of drugs (0–100 μ M) 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 was 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 phosphorescence anisotropy (TPA)

Phalloidin-stabilized EriA-F-actin was diluted in F-Mg buffer to 2.0 μ M, and compounds were added at specified concentrations (0–100 μ M). To maximize phosphorescence signals and prevent photobleaching of the dye, oxygen was removed from the sample by 5-min incubation with glucose oxidase (55 μ g/ml), catalase (36 μ g/ml), and glucose (45 μ g/ml). Phosphorescence was measured at 25°C as described previously (10). EriA actin was excited with a vertically polarized 1.2-ns pulse from a FDSS 532-150 laser (CryLas) at 532 nm, operating at a repetition rate of 100 Hz. Phosphorescence emission was 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 μ s/channel. The TPA decay was calculated as $r(t) = [I_v(t) - G I_h(t)] / [I_v(t) + 2 G I_h(t)]$, where $I_v(t)$ and $I_h(t)$ 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 minutes. TPA decays were analyzed by calculating the final anisotropy (r_∞), defined as the average value of r in the time window from 400 to 500 μ s, 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 was calculated as described previously (41)

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Potential conflict of interest - DDT holds equity in, and serves as President of, Photonic Pharma LLC. This relationship has been reviewed and managed by the University of Minnesota. Photonic Pharma had no role in this study.

Author contributions - PG, EP, and DDT designed the research. PG and EP prepared samples, performed biochemical experiments, and analyzed the data. KCP designed, constructed, and maintained the FLTPR. PG, EP, and BDG acquired and analyzed fluorescence data. PG, EP, and DDT wrote the paper.

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Table 1 EC₅₀ values(μM)

Compound	EC ₅₀ (FRET)	EC ₅₀ (ATPase) Skeletal actoS1	EC ₅₀ (ATPase) Cardiac actoS1
Honokiol	44 ± 2	>100	>100
Tegaserod	29 ± 2	>80	20.2 ± 5.1
Thioridazine	21 ± 3	25 ± 3.0	29± 6
Flutamide	15 ± 2	n.d	n.d
Phenothiazine	7 ± 2	n.d	n.d.
Fluphenazine	25 ± 4	12.9 ± 3.9	11.0± 3.2
Dantrolene	48 ± 4	n.d	n.d
Novantrone	5.4 ± 0.7	11.3 ± 3.5	11.0 ± 2.0
Carvedilol	48 ± 5	n.d	n.d
Mefloquine	21 ± 2	n.d.	n.d

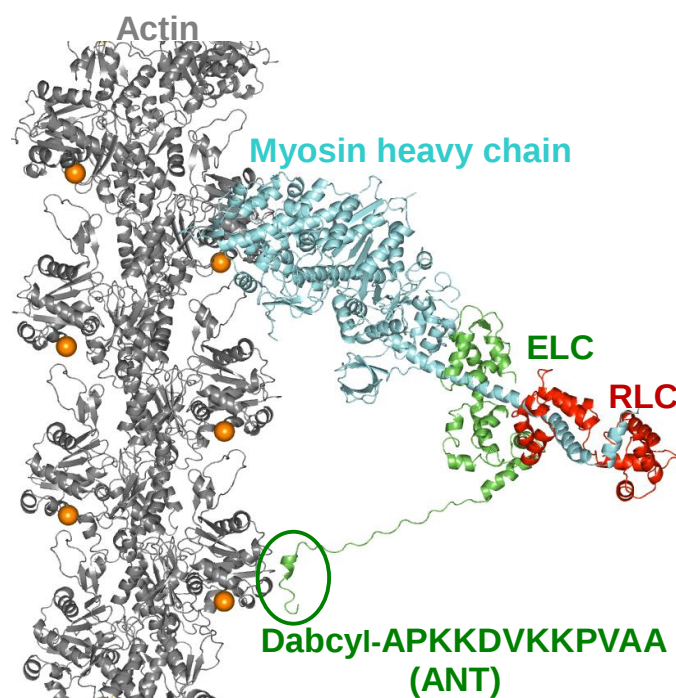


Fig. 1. Model of actin-myosin complex with skeletal myosin S1 (heavy chain blue, ELC green, RLC red) strongly bound to actin (gray) (19). Orange spheres show the labeling site on actin. The green circle indicates location of the Dabcyl-labeled N-terminal peptide (ANT).

High-throughput FRET assay detecting actin-myosin modulators

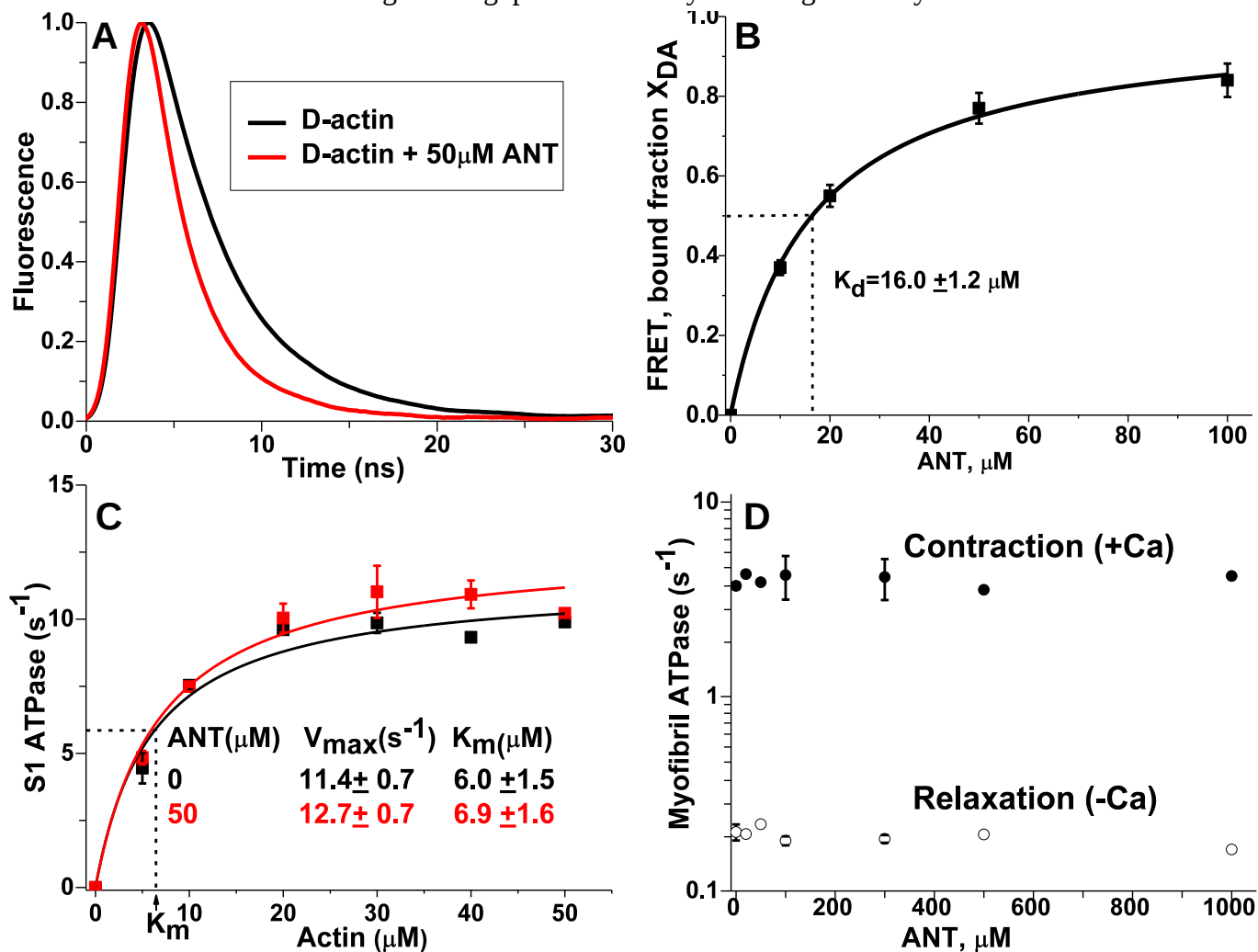


Fig. 2. A: TR-FRET. Time-resolved fluorescence of 2 μM donor-labeled actin (D-actin) in the absence (black) and presence (red) of 50 μM acceptor-labeled N-terminal peptide (ANT). B: Binding of ANT to D-actin, calculated from TR-FRET data in A. C: Actin-activated ATPase activity of skeletal muscle S1A1 (100% A1) in the absence (black) and in the presence of 50 μM ANT (S1A1 = 0.15 μM). D: Myofibrillar ATPase activity vs. [ANT] during relaxation (open circles) and contraction (closed circles).

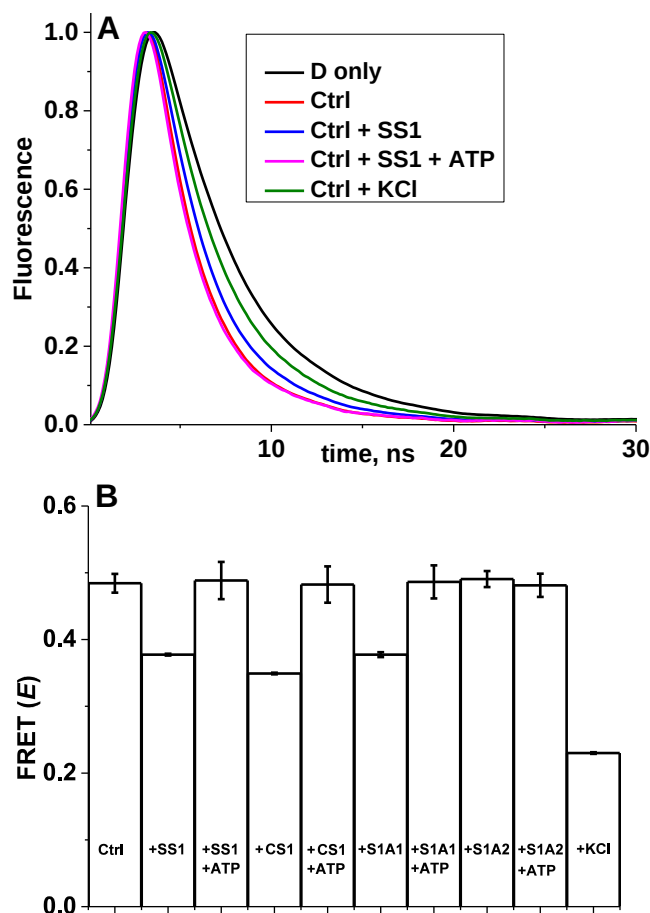


Fig. 3. Actin-ANT FRET. A: Representative fluorescence decays of donor-labeled actin (2 μ M) in the absence (D only, black, same as D-actin in Fig 2A) and presence of 50 μ M ANT (Ctrl, red, same as D-actin+50 μ M ANT in Fig 2A) and after addition to Ctrl of saturating (5 μ M) skeletal S1 (blue), S1+ saturating (3 mM) ATP (magenta), or 0.1 M KCl (green). The D only (black) and Ctrl (red) decays are included in the present figure for visualization and comparison purpose. B: FRET between actin and ANT in the absence (Ctrl) and presence of different isoforms of S1 (skeletal SS1, cardiac CS1, skeletal S1A1, skeletal S1A2), without and with 3 mM ATP and 0.1 M KCl.

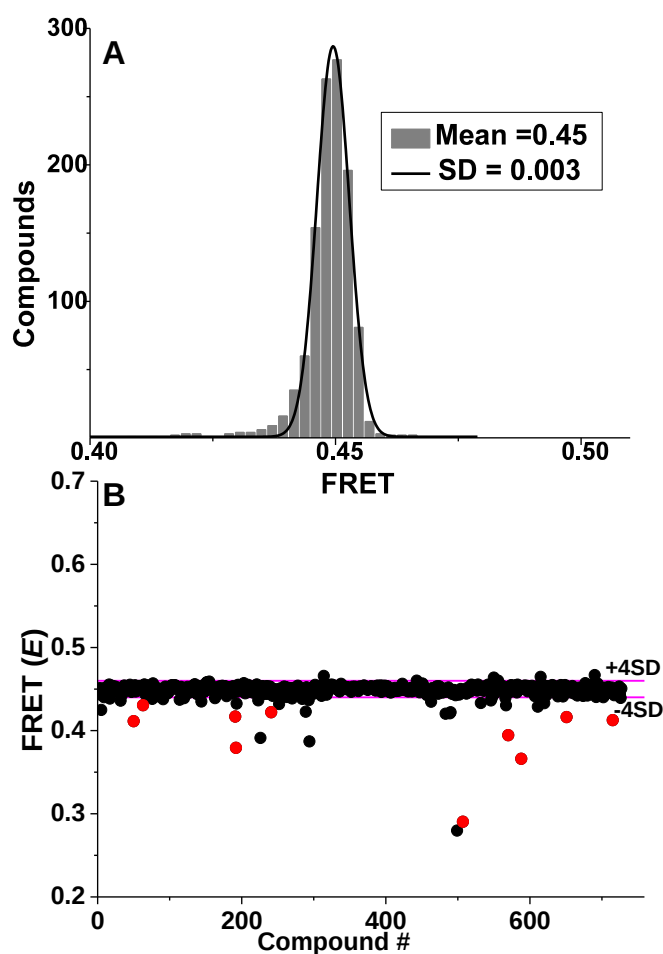


Fig. 4. TR-FRET based HTS of NCC library for compounds that modulate actin-ANT FRET. A. Histogram plots of all compounds from the NCC screen after removal of fluorescent compounds show an average FRET efficiency of 0.45 ± 0.003 . B. FRET from a representative NCC screen with Hit threshold (>4 SD of mean) indicated by magenta lines. Reproducible Hits from triplicate screens are shown in red.

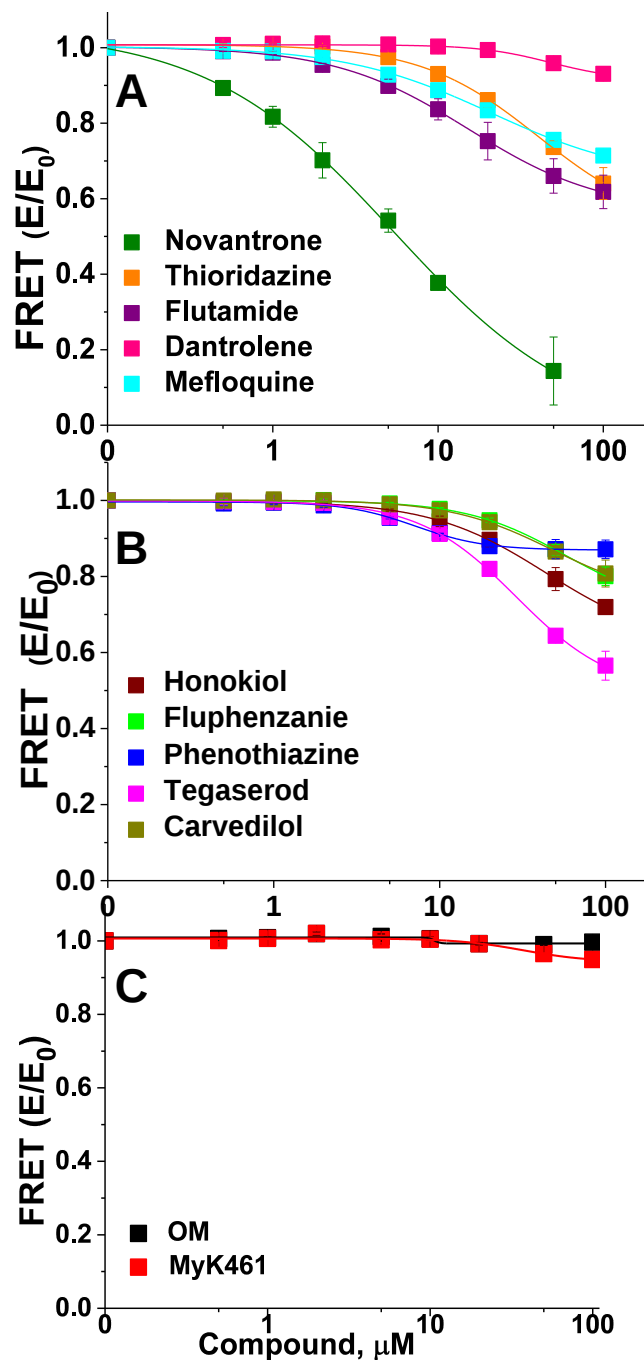


Fig. 5. Concentration response of reproducible Hit compounds on FRET. A and B. Hit compounds decreased FRET in a concentration-dependent manner. C. Myosin-specific compounds, OM and MYK461 did not show concentration dependence (control experiment).

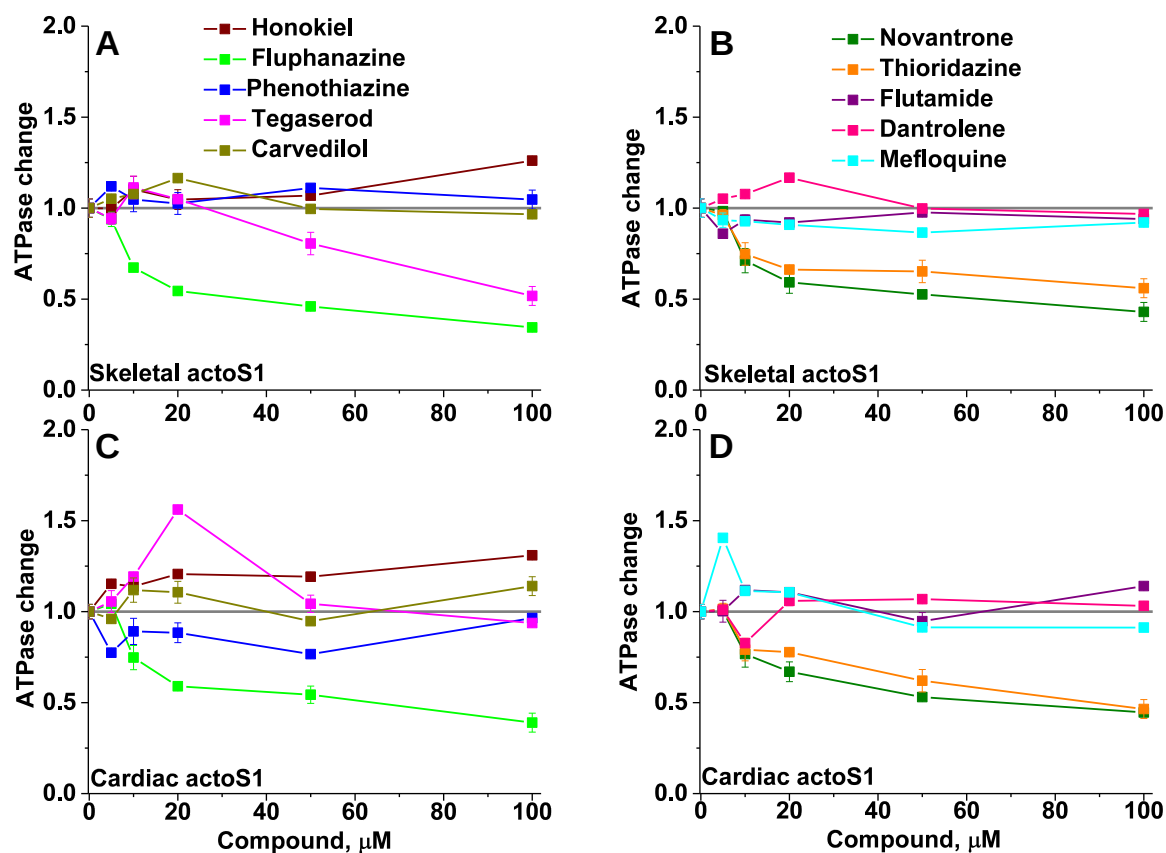
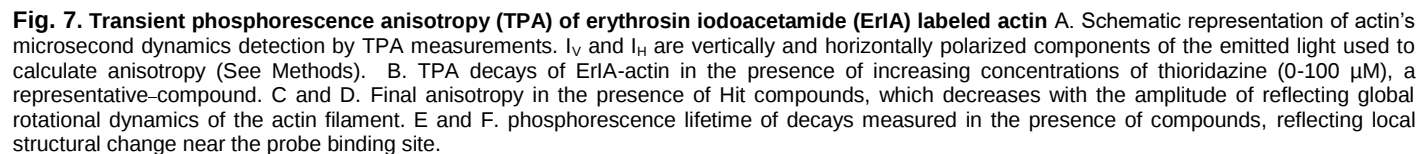


Fig. 6. Concentration-dependence of the ATPase activity of actoS1. A and B. Skeletal actoS1. C. and D. Cardiac actoS1. Data is normalized to the value of the ATPase in the absence of the compounds.



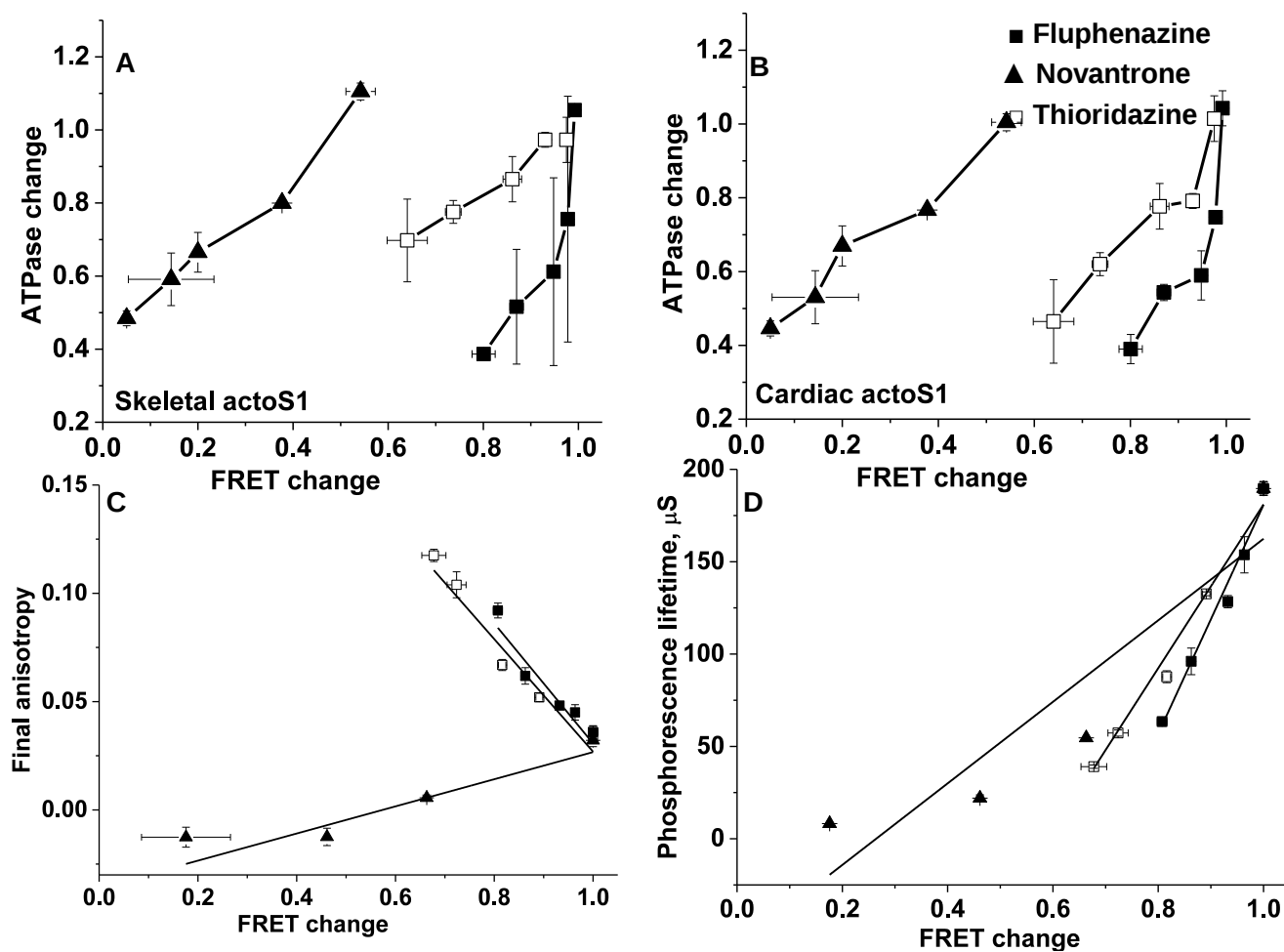


Fig. 8. Structure-function correlation. A. ATPase vs FRET change for skeletal actoS1. B. ATPase vs FRET change for cardiac actoS1. Significant-inhibitors show that the FRET change is proportional to ATPase change. C. FRET change vs final anisotropy. D. FRET change vs phosphorescence lifetime. Compound associated change in FRET is proportional to actin anisotropy, suggesting the compound related change in actin structure.

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

Piyali Guhathakurta, Ewa Prochniewicz, Benjamin D. Grant, Kurt C. Peterson and David D.
Thomas

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