

A small-molecule inhibitor of skeletal muscle myosin II

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We screened a small-molecule library for inhibitors of rabbit muscle myosin II subfragment 1 (S1) actin-stimulated ATPase activity. The best inhibitor, *N*-benzyl-*p*-toluene sulphonamide (BTS), an aryl sulphonamide, inhibited the Ca²⁺-stimulated S1 ATPase, and reversibly blocked gliding motility. Although BTS does not compete for the nucleotide-binding site of myosin, it weakens myosin's interaction with F-actin. BTS reversibly suppressed force production in skinned skeletal muscle fibres from rabbit and frog skin at micromolar concentrations. BTS suppressed twitch production of intact frog fibres with minimum alteration of Ca²⁺ metabolism. BTS is remarkably specific, as it was much less effective in suppressing contraction in rat myocardial or rabbit slow-twitch muscle, and did not inhibit platelet myosin II. The isolation of BTS and the recently discovered Eg5 kinesin inhibitor, monastrol¹, suggests that motor proteins may be potential targets for therapeutic applications.

Small molecules that can enter cells and perturb the function of a specific target protein are useful tools in basic cell biology research and as leads for developing therapeutic agents. Natural-product toxins that target actin and tubulin with high affinity and specificity are key tools in cytoskeleton research. However, we currently lack small-molecule tools for most other cytoskeletal proteins^{2–5}.

Here we report a search for small-molecule inhibitors of myosin II, a motor protein involved in muscle contraction and many cellular processes, including cytokinesis and cell migration⁶. Previously described myosin inhibitors suffer from either questionable specificity or a lack of cell permeability^{7–10}. The low affinity reagent 2,3-butane dione monoxime (BDM) targets muscle myosin II (ref. 11), but it can also affect Ca²⁺ regulation^{12,13}, and we have found that it does not inhibit nonmuscle myosin II (A.F.S., A.C. and T.J.M., unpublished observation). The cytoskeleton and cell-motility fields would benefit considerably from cell-permeable, specific, small-molecule inhibitors of myosin II isoforms with biochemically defined modes of action.

We screened a small-molecule library and identified a specific inhibitor of rabbit muscle myosin subfragment 1 (S1) actin-stimulated ATPase activity. Extrapolating from the myosin inhibitor reported here, and the Eg5 kinesin inhibitor monastrol¹, we surmise that motor proteins in general may be excellent potential targets for small molecules.

We set up a high-throughput luminescence assay to measure actin-activated ATPase activity (Fig. 1a, b). S1 from rabbit muscle was mixed with polymerized actin and distributed into wells of assay plates without nucleotides. Compounds from a chemical library were transferred into the wells with a pin array. The reaction

was initiated with MgATP. Assay plates were incubated until >95% of the added ATP in control wells was hydrolysed. The remaining ATP was determined by distributing a solution containing luciferin and luciferase into the wells and measuring the luminescence signal. We screened 16,300 compounds from a commercial chemical library (Diverset E; Chembridge Inc.), and found four compounds from two structural classes (aryl sulphonamides (1,2) and hydroxycoumarins (4,5); Fig. 1c) that reproducibly and strongly inhibited the myosin S1 ATPase activity. We focused on the aryl sulphonamides because they were more potent and easier to synthesize.

We prepared a mini-library of 240 aryl sulphonamides related to (and containing) the two best hits from our library screen, and tested them in the myosin ATPase assay (Fig. S1 in Supplementary Information). The hits from this mini-library clustered tightly around the original library hits, and none of the new compounds were significantly more potent than the compound originally found (Fig. S1, position 3H). The systematic name for 3H is *N*-benzyl-*p*-toluenesulphonamide (BTS), and it is commercially available (Sigma-Aldrich Rare Chemicals, catalogue number S949760). By running our standard luminescence assay with dilutions of BTS, we determined an IC₅₀ for the actin-activated ATPase of ~5 μM (data not shown). Our mini-library identified a useful negative control compound (3 in Fig. 1c and position 5C in Fig. S1). 5C is an isomer of BTS that had no detectable activity in the luminescence assay at 300 μM.

To test whether BTS targets myosin or actin, we measured its effect on the myosin S1 Ca²⁺-stimulated ATPase in the absence of actin (Fig. 2a). BTS inhibited this ATPase activity with an IC₅₀ of ~5 μM, which is similar to its IC₅₀ for inhibition of actin-stimulated myosin S1 ATPase activity. BTS must, therefore, bind to, and inhibit, skeletal myosin S1. To test whether BTS inhibits the motor activity of skeletal muscle myosin, we performed a gliding-filament assay using heavy meromyosin (HMM). At 2 μM BTS, sliding velocity was more than halved compared with untreated control (control 2.89 ± 0.07 μm s⁻¹, BTS 1.33 ± 0.11 μm s⁻¹), and reduced to 3.5% of the control at 20 μM (0.10 ± 0.04 μm s⁻¹). The control compound, 5C, had no effect at 20 μM (2.69 ± 0.04 μm s⁻¹). After BTS was washed out of the chamber, gliding motility resumed at the pre-inhibition rate. BTS therefore blocks the motor activity of myosin at a concentration in the same range as its IC₅₀ in the solution ATPase assays; this inhibition is rapidly reversible.

We next probed the mechanism by which BTS inhibits myosin. To determine if BTS competes for the nucleotide-binding site, we performed a competition binding assay between ADP and a fluorescent ADP derivative, 3'-O-(*N*-methylantraniloyl)-ADP (Mant-ADP; Fig. 2b)¹⁴. Adding increasing concentrations of ADP reduced the fluorescence of Mant-ADP, indicating competition between the two nucleotides. In contrast, adding BTS in large excess over its IC₅₀ did not decrease the fluorescence. These data imply that Mant-ADP

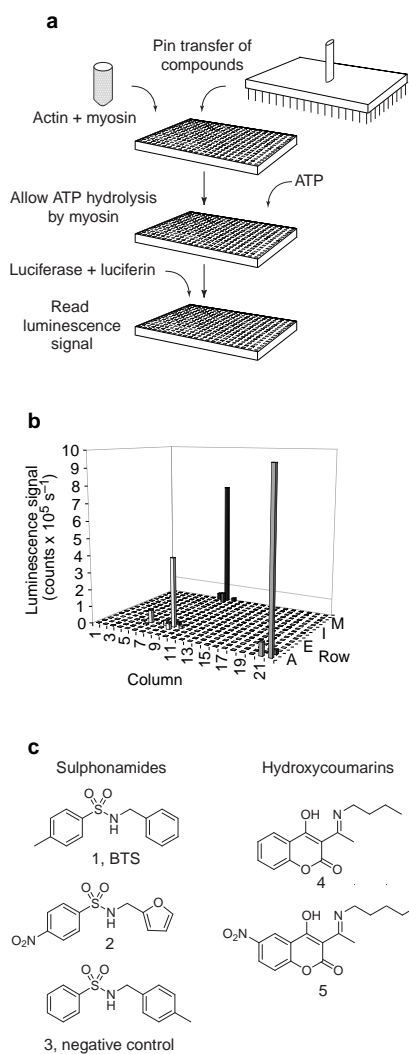


Figure 1 Screen for inhibitors of myosin II. **a**, A schematic of the luciferase-based assay for chemical inhibition of skeletal myosin S1 ATPase. **b**, Screening data from compound plate 47 shows two potential inhibitors of myosin ATPase. Compounds were present at $\sim 100 \mu\text{M}$ in wells A1 through P20. A positive control of 10 mM BDM was included in well A22. **c**, Positive hits from myosin screen. Two sulphonamides (**1** and **2**) and two hydroxycoumarins (**4** and **5**) were identified. BTS is compound **1** and the inactive isomer of BTS, 5C, is compound **3**.

can bind to myosin in the presence of concentrations of BTS that are saturating in the ATPase inhibition assays, indicating that nucleotides and BTS bind to different sites.

To determine the effect of BTS on F-actin binding by myosin, we used rabbit skeletal muscle HMM in a sedimentation assay (Fig. 2c). In a solution lacking nucleotide, all the full-length HMM cosedimented with F-actin in the absence or presence of 100 μM BTS. The sensitivity of this assay may not, however, be sufficient to detect a change in the strong affinity of nucleotide-free myosin for F-actin. When 1 mM ATP was added, very little HMM cosedimented with actin whether or not BTS was present. In the presence of 1 mM ADP without BTS, most of the HMM cosedimented with actin. The addition of BTS to the actomyosin ADP mixture dissociated the HMM. BTS therefore inhibits binding of actin by myosin-ADP. Consistent with these observations, kinetic studies show that, in the presence of BTS, the affinity of ADP-S1 for actin

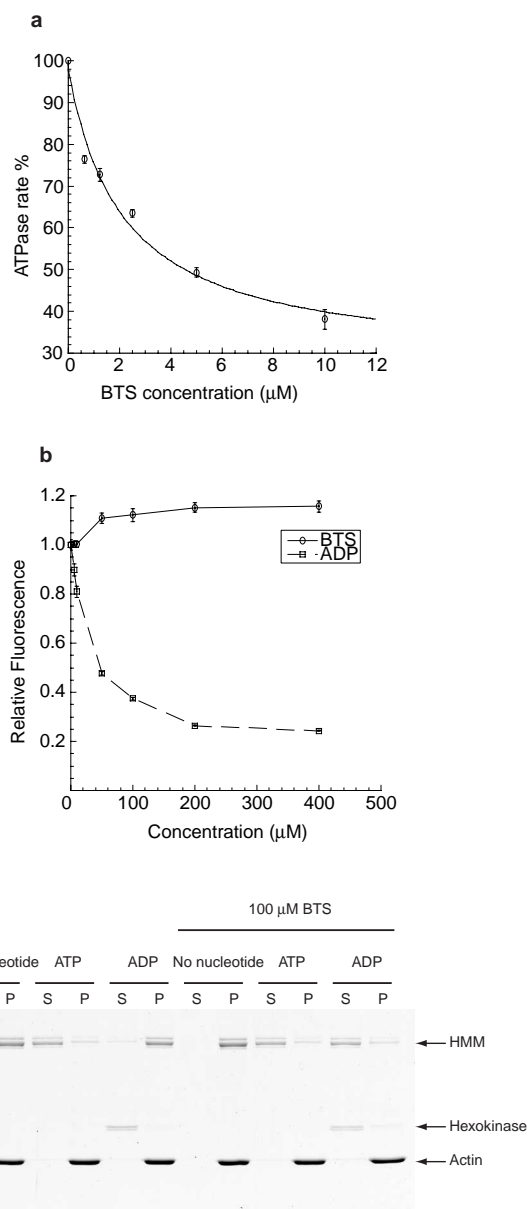


Figure 2 Inhibition of skeletal myosin by BTS. **a**, BTS inhibits the Ca^{2+} -stimulated ATPase activity of myosin S1 with an IC_{50} of $\sim 5 \mu\text{M}$. **b**, BTS does not compete with ADP binding to myosin. Relative fluorescence intensity of Mant-ADP is shown with increasing concentrations of ADP or BTS. The fluorescence intensity was normalized to buffer controls lacking ADP or BTS (see Methods). **c**, BTS releases myosin from actin in the presence of ADP. Supernatant and pellet from sedimentation are labelled S and P respectively. Relative migration of molecular mass standards is shown. Sedimentation in the absence of F-actin is shown in Fig. S2.

is decreased, the maximum actin-activated ATPase rate is reduced, and the actin concentration for half maximal activation of the ATPase activity is increased (M. A. Shaw, E. M. Ostap and Y. E. Goldman, personal communication).

To test the effect of BTS on the fully assembled myofibril array, we examined the contractile properties of skinned muscle fibre preparations (Fig. 3a–c). In glycerol-extracted fibres from rabbit psoas muscle, BTS inhibited isometric Ca^{2+} -activated tension with an IC_{50} of $\sim 3 \mu\text{M}$ (Fig. 3a). At 100 μM BTS, tension decreased

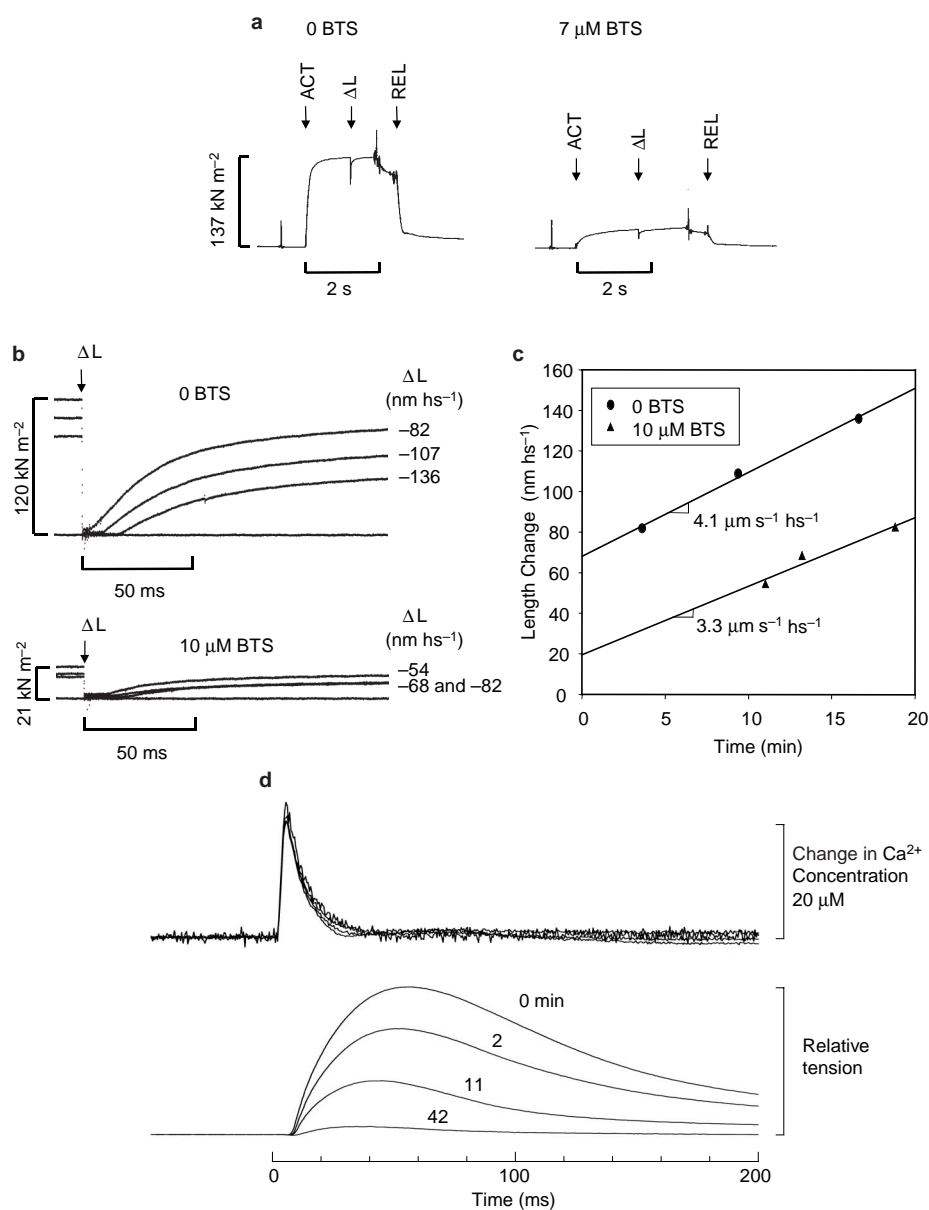


Figure 3 **BTS inhibition of muscle contractile properties.** **a**, Strip-chart recordings of isometric tension for a skinned fibre of rabbit psoas muscle without or with BTS. Changes into ACT and REL solutions are as indicated. Fibre stiffness was measured with small length steps (ΔL) during full isometric tension. The deflections in the traces before ACT and REL are mechanical artifacts caused by passage of the fibre through a solution/air interface during solution change. **b**, Unloaded shortening velocity of a skinned fibre of rabbit psoas muscle without or with BTS. The arrows mark the timing of the length steps slackening the fibre during full isometric contractions. The step amplitudes are indicated next to the tension transients in nm per half sarcomere (nm/hs). The bottom trace at each concentration is the relaxed tension level. A line fitted to the initial slope of tension redevelopment was used to estimate the slack time. **c**, The slopes of the

lines in **b** fitted to a plot of amplitude versus slack time give the maximum shortening velocity (V_{max}) for each BTS concentration. **d**, Effect of 5 μM BTS on a single intact frog fibre stretched to a sarcomere length of 3.3 μm . The upper panel shows transient calcium concentration changes and the lower panel simultaneous twitch tension responses from four successive measurements. The numbers next to the tension traces indicate the time, in minutes, that the fibre was exposed to BTS. The peak twitch tension remaining after 55 min (data not shown) was 4.6% of that observed initially. After re-exposure of the fibre to Ringers without BTS, the tension response showed partial recovery. The most likely explanation why full recovery was not observed is that BTS bound to the lucite walls of the chamber preventing full washout.

to <3% of its control value. The 5C isomer of BTS suppressed tension by only 15% at 500 μM .

Unloaded shortening velocity of rabbit fibres was determined by applying large (4–10%) length releases to slacken a fibre (Fig. 3b,c) and measuring the time required to take up this slack¹⁵. The slopes of the lines fitted to the amplitude of shortening versus slack times

give the shortening velocity. Although 10 μM BTS suppressed isometric tension by ~82%, unloaded shortening (triangles in Fig. 3c) was suppressed by only ~19% of that in the absence of BTS (circles). At partial tension suppression by BTS, some cross-bridges attach to the thin filament producing translocation at the normal rate. This provides strong evidence against formation of rigour-type

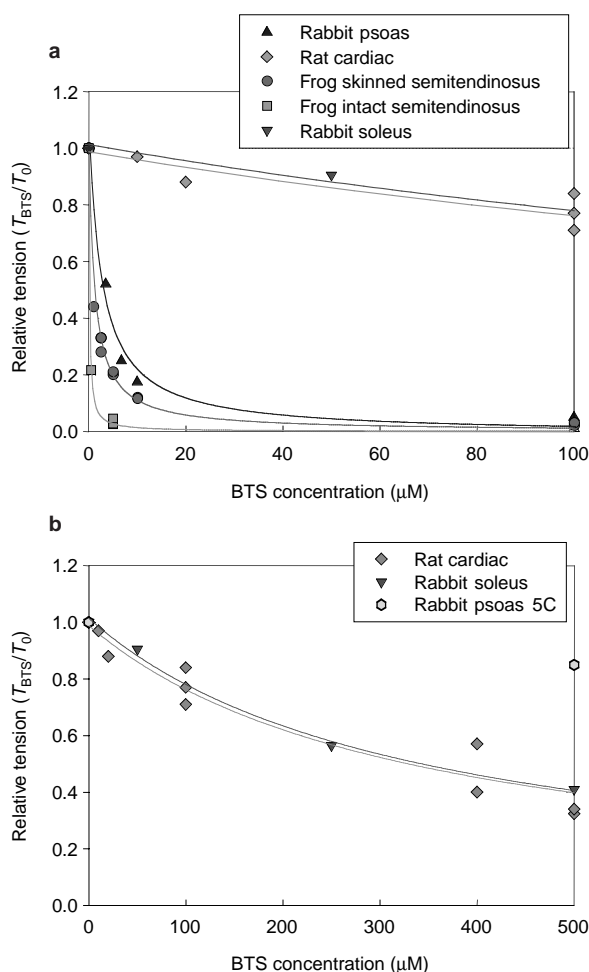


Figure 4 Plots of tension suppression by BTS for all fibre types tested. Each symbol/shade represents a different fibre type (see keys). The following equation $T_{BTS} = T_0(K_i / ([BTS] + K_i))$ was used to fit data from each fibre type. **a** illustrates the high affinity of fast skeletal muscle for BTS compared to soleus and cardiac muscle preparations. The following values of K_i were obtained from the fits: 3 μM , rabbit psoas, 320 μM , cardiac and soleus, 1 μM , skinned frog, and 0.14 μM , intact frog. In **b**, data for cardiac and soleus preparations, including higher concentrations are plotted. Slight suppression of a rabbit psoas fibre by the 5C isomer of BTS (500 μM) is also shown.

attachments in the presence of BTS. In fact BTS decreased rigour tension and stiffness even in the absence of ATP and removal of the BTS led to partial recovery of rigour tension (data not shown).

As the BTS concentration was raised, stiffness, tested by application of quick length changes in active contraction¹⁶, declined approximately in proportion to the steady tension ($IC_{50} = 7 \mu\text{M}$). The recovery transients initiated by the quick steps were qualitatively similar to those in the absence of BTS. These results indicate that BTS reduces the number of attached myosin heads without markedly altering the kinetic properties of the remaining attachments. For the rabbit fibres, the suppression of tension and stiffness was rapidly reversible (<5 min) if the fibre was washed in a BTS-free relaxing solution.

In mechanically skinned frog semitendinosus fibres, BTS also suppressed isometric Ca^{2+} -activated tension ($IC_{50} = 1 \mu\text{M}$, Fig. 4), but in glycerol-extracted soleus fibres of rabbit and chemically skinned trabeculae and papillary muscle from rat hearts BTS was

much less effective ($IC_{50} = 320 \mu\text{M}$; Fig. 4b). Tension suppression in the myocardial and soleus preparations was prompt (wash in and wash out <4 min), but the frog skinned fibres reacted much more slowly. Full suppression occurred in >25 min and full recovery required multiple contractions or long contraction times.

The selectivity of BTS for fast skeletal versus slow or cardiac muscle prompted further biochemical tests of specificity. BTS at 100 μM did not inhibit gliding motility of kinesin, or the nucleotide-binding enzymes, pyruvate kinase, lactate dehydrogenase and firefly luciferase. To test specificity between myosins, we prepared human platelet myosin II and its activator, myosin light chain kinase, and assayed the actin-stimulated ATPase activity of this myosin. Remarkably, 100 μM BTS did not inhibit platelet myosin II (data not shown).

To test whether BTS would permeate the membrane of an intact cellular preparation and specifically inhibit contraction, intact, live single fibres from frog twitch muscle were equilibrated in standard Ringers solution at 16 °C, injected with a fluorescent Ca^{2+} indicator, and electrically stimulated to produce twitches (Fig. 3). BTS (5 μM) suppressed twitch contraction by 97% after ~60 min of incubation without affecting the calcium transient. Twitch force was inhibited 50% by 0.14 μM BTS (Fig. 4a). At $\geq 100 \mu\text{M}$ BTS, the refractory period for repetitive stimulation was lengthened, indicating action potential prolongation. At 5 μM BTS, the half-time of twitch tension suppression was 5–10 min. This onset is probably not limited by diffusion through the surface membrane because skinned frog fibres also reacted to BTS slowly. Curves of tension inhibition for the five fibre types by BTS and, for psoas muscle the 5C isomer, are shown in Fig. 4.

The substituted aromatic structures and relatively modest IC_{50} values of our myosin inhibitors resemble the lead compounds that often form the starting point for therapeutic drug development. To test the feasibility of compound optimization with limited resources, we used parallel synthesis to make a small library related to our sulphonamide hits. None of the new compounds was more potent than BTS, showing that the steric requirements for binding of BTS to myosin II are quite strict. BTS inhibits rabbit fast muscle myosin II at least 100 \times more potently than human nonmuscle, rabbit slow muscle and rat cardiac muscle myosin II. These myosins have greater than 50% sequence identity in their motor domains, very similar folds, and very similar biochemical mechanisms^{17,18}. Thus, BTS acts with remarkable specificity consistent with the restricted steric requirements for binding to the fast muscle myosin.

BTS binding weakens the affinity of myosin for actin, leading to dissociation of myosin in the presence of ADP. BTS might bind at the nucleotide-binding site, but our competition binding data with Mant-ADP argue against this. BTS might compete directly with actin at the actin-binding site or might bind to a third site on myosin II and allosterically weaken actin binding. Our current data do not distinguish between these possibilities. We favour the allosteric model because BTS seems too small to compete directly with actin, and inhibition of the Ca^{2+} -stimulated myosin ATPase is actin-independent.

The experiments on muscle fibre preparations support the conclusion that BTS weakens actomyosin affinity. A few percent of rigour cross-bridges would markedly slow down maximum shortening velocity; but BTS suppressed isometric force much more strongly than active sliding velocity (Fig. 3b, c). During isometric force suppression by BTS, the tension transients initiated by quick length changes were relatively normal. Furthermore, BTS directly caused detachment of rigour cross-bridges. Thus BTS acts to inhibit myosin head attachment to actin but those heads that remain uninhibited at moderate BTS concentrations have normal kinetics.

Soleus and cardiac muscle express the slow (β) isoform of myosin II, and these muscle types were much less sensitive to BTS than fast muscle fibres from rabbit or frog (Fig. 4). BTS inhibition is

therefore specific to myosin rather than actin. In the intact frog single fibres, the suppression of twitch tension at concentrations ($<5 \mu\text{M}$) that caused little alteration of the 'calcium transient' (Fig. 3c) indicates that BTS is membrane permeable and specific for the contractile apparatus.

There are several practical applications for BTS in studying skeletal myosin. BTS might help in dissecting the detailed mechanism by which ATP hydrolysis powers force production — we envisage that using BTS will facilitate structural analysis of an intermediate in the myosin enzymatic cycle not otherwise accessible. An agent that prevents muscle contraction by specifically targeting myosin II will also be useful to muscle physiologists. Finally, muscle relaxants have multiple medical uses¹⁹, and no current drug targets specific myosins.

We now have two examples of small molecules that specifically target motor proteins, BTS and the Eg5 inhibitor monastrol¹. We conclude that motors are accessible drug targets, which could have significant implications for both basic cell biology research and therapeutics. Motors function in most cells and are involved in tissue-specific physiology^{20,21}. Specific small-molecule inhibitors will be key tools for analysing the function of these motors. Motors for which loss of function has therapeutic benefit are promising drug targets. To achieve our goal of finding small-molecule tools for cytokinesis and cell motility research, we are now screening small-molecule libraries for inhibitors of nonmuscle myosin II. □

Methods

Protein purification

Myosin was purified from fresh rabbit skeletal muscle according to Margossian and Lowy²². The S1 fragment of myosin was generated by papain (Sigma, St. Louis, MO) digestion as described previously²² followed by ion-exchange chromatography on Q-sepharose (Pharmacia Biotech, Piscataway, NJ). HMM was made by digesting myosin with chymotrypsin²² and further purified by ion-exchange chromatography on DEAE-sephadex. Actin was purified from rabbit skeletal muscle as previously reported²³ and polymerized to F-actin in 50 mM KCl, 1 mM MgCl₂, and 1 mM ATP.

High-throughput screening of chemical libraries

Five microlitres of a mixture of 0.1 mg ml⁻¹ myosin S1, 0.4 mg/ml F-actin, 1 mM DTT in assay buffer (25 mM HEPES pH 7.6, 50 mM KCl, 2 mM MgCl₂) was dispensed into a 384-well white microplate (Greiner Labortechnik, Frickenhausen, Germany). Chemical compounds stored in DMSO at a concentration of 5 mg ml⁻¹ were transferred into 384-well plates using a steel pin array mounted on an X,Y,Z robot to give a final concentration of $\sim 100 \mu\text{M}$. The reaction was initiated by adding 5 μl per well of 200 μM ATP then incubated for 1 h at ambient temperature (20–24 °C). To measure the remaining ATP, 40 μl of development solution (0.5 mM luciferin, 1.25 $\mu\text{g ml}^{-1}$ luciferase, 25 mM Tricine pH 7.8, 5 mM MgSO₄, 100 μM EDTA and 1 mM DTT; Molecular Probes, Eugene, Oregon) was added to each well. The luminescence signal in each assay well was then measured using a plate reader (PerkinElmer, Shelton, CT) within 15 min.

Construction of the sulphonamide mini-library

To a stirred solution of 100 μmoles of each sulphonyl chloride (Fig. S1) dissolved in 0.5 ml of dichloromethane, 0.5 ml of a mixture of 115 μmoles of each amine (Fig. S1) and 115 μmoles of diisopropylethylamine in dichloromethane was added. The reaction mixtures were stirred for 2 h at room temperature. To terminate the reaction, $\sim 0.1 \text{ g}$ of aminomethyl polystyrene resin (Sigma) followed by 0.1 g of amberlyst resin was added to each mixture and incubated for 10 min. Reaction mixtures were then filtered and dried. Thin-layer chromatography was used to verify the reaction and reverse phase HPLC (C-18) and mass spectrometry were performed on random samples for confirmation. Each compound was resuspended in DMSO to a final concentration of $\sim 20 \text{ mg ml}^{-1}$. For activity testing, the compounds were distributed into 384-well plates at 5 mg ml⁻¹ in DMSO.

In vitro motility

Actin filament sliding on myosin S1 and on HMM was assayed according to Sellers *et al.*²⁴ with the following modifications. HMM was adsorbed to nitrocellulose-coated coverslips in flow-cell chambers at a concentration of 0.1 mg ml⁻¹. Chambers were blocked with 0.5 mg ml⁻¹ casein, and then F-actin that had been stabilized with TRITC-phalloidin was added at a concentration of 20 nM. Motility was initiated by adding 1 mM ATP in assay buffer (20 mM KCl, 10 mM MOPS pH 7.2, 5 mM MgCl₂, 0.1 mM EGTA, 10 mM DTT, 2.5 mg ml⁻¹ glucose, 0.1 mg ml⁻¹ glucose oxidase, 0.02 mg ml⁻¹ catalase) and filament sliding was recorded at 10 s intervals using a 1.4 NA 60 \times lens (Nikon) and a cooled charge-coupled display (CCD) camera (Princeton Instruments). For control, 50, 20 μM BTS and 2 μM BTS the number of filaments tracked were 63, 27, 51 and 54 respectively in 8, 3, 6 and 5 individual experiments. Rates are expressed as velocity in $\mu\text{m sec}^{-1} \pm$ standard error.

Measurement of Ca²⁺-stimulated S1 ATPase

Myosin S1 was diluted to 0.025 mg ml⁻¹ in reaction buffer (200 mM KCl, 50 mM Tris pH 7.5, 2.5 mM CaCl₂, 1 mM MgCl₂, 1 mM DTT, 0.2 mM 2-amino-6-mercapto-7-methyl-purine riboside (MESG) and 10 U ml⁻¹ purine nucleoside phosphorylase (PNP) (Molecular Probes)). The reaction

was initiated by adding ATP to 100 μM and absorbance at 360 nm was read every 5 s. Inhibition was assayed at 0, 0.625, 1.25, 2.5, 5 and 10 μM BTS.

Mant-ADP binding assay

0.5 μM myosin S1 was mixed with 5 μM Mant-ADP (Molecular Probes) in assay buffer (25 mM HEPES pH 7.6, 50 mM KCl, 2 mM MgCl₂). Solutions of ADP, BTS and 5% DMSO, or 5% DMSO were added to the mixture of myosin S1 and Mant-ADP to give final ADP or BTS concentrations of 5, 10, 50, 100, 200 or 400 μM . Mant-ADP fluorescence was excited at 290 nm and assayed at 448 nm. Fluorescence values in the presence of ADP or BTS were divided by the fluorescence of the buffer control to correct for dilution effects.

Cosedimentation of F-actin and HMM

G-actin (35 μM) was polymerized in F-buffer (100 mM KCl, 25 mM Tris pH 7.0, 1 mM MgCl₂, 0.2 mM CaCl₂, 0.5 mM DTT, 1 mM ATP) for 45 min at 37 °C. F-actin was stabilized by adding phalloidin to 45 μM and was dialysed against spindown buffer (200 mM KCl, 50 mM Tris pH 7.0, 2 mM MgCl₂, 1 mM DTT, 50 mM glucose) to remove residual ATP. HMM (0.5 μM) was incubated with or without F-actin (1.5 μM) in binding buffer for 15 min at room temperature, then 10 μl of the mixture was distributed into separate tubes. ADP (50 mM) was preincubated with 50 U ml⁻¹ hexokinase (Sigma) in spindown buffer for 20 min at 37 °C to hydrolyse contaminant ATP. To the actin–myosin mixture, an equal volume of binding buffer alone or binding buffer containing either 2 mM ATP or 2 mM ADP and 0.2 U hexokinase was added. BTS dissolved in DMSO or DMSO alone was added at the time of nucleotide addition to yield a final concentration of 100 μM BTS or 0.1% DMSO. The reaction mixture was incubated at 22 °C for 10 min then centrifuged at 217000 g (75000 rpm) for 10 min in a TLA-100 rotor (Beckman). The supernatants and pellets were solubilized in SDS-PAGE sample buffer, separated on a 10% SDS-PAGE gel and stained with Coomassie brilliant blue.

Fibre preparation

Relaxed bundles of rabbit psoas or soleus muscle were chemically skinned in a 50% glycerol-extraction solution (5 mM MgATP, $<10^{-8} \text{ M Ca}^{2+}$, -20°C)²⁵ to remove the sarcolemma, leaving the contractile apparatus intact. Single fibres were teased from the bundles under silicone oil (12 °C) and rinsed with glycerol-free relaxing solution. Aluminum foil clips (T-clips) were fastened around the ends of the fibres to secure the fibre between hooks on a force transducer and a servomotor (200 μs length changes)²⁶.

Strips of rat cardiac trabeculae and papillary muscles were skinned with Triton-X 100 detergent in a high ATP, low Ca²⁺, relaxing solution. Nylon sutures were fastened to the ends of the strips and then mounted onto the experimental apparatus to obtain force measurements.

Single fibres of the semitendinosus muscle of frogs (*Rana temporaria*) were isolated under silicone oil (4 °C). Using an electrolytically sharpened tungsten needle, the sarcolemma and basal lamina were peeled off in relaxing solution²⁷.

Intact single fibres were dissected from the frog semitendinosus or ilio-fibularis muscle in Ringers solution (120 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 5 mM PIPES pH 7.1 at 16 °C). The tendons were used to secure the fibres to the experimental apparatus. At one end a gauge recorded twitch tension production. Each fibre was microinjected with a nonperturbing concentration of fura2/1 (a low-affinity Ca²⁺ indicator) to measure the change in myoplasmic free Ca²⁺ concentration during stimulated twitches²⁸.

Activation of skinned muscle preparations

The skinned fibre preparations were all activated using the same protocol. Solutions were exchanged by keeping the fibre bundle stationary and translating a trough assembly to a new 60 μl chamber with the fresh solution. The fibres/bundles were first equilibrated in a relaxing solution (REL; 100 mM Tes, 5 mM MgATP, 1 mM Mg²⁺, 20 mM EGTA, 10 mM glutathione, 200 mM ionic strength, $<10^{-8} \text{ M free Ca}^{2+}$, pH 7.1 at 20 °C). The fibre was then incubated for at least 4 min in a preactivating solution (PRE, same as REL except 0.1 mM EGTA, 19.9 mM HDTA). The preparations were then activated (ACT) in the presence of $\sim 32 \mu\text{M}$ free Ca²⁺ developing and maintaining tension for $<30 \text{ s}$. BTS, dissolved in DMSO, was added to the solutions. The frog skinned-fibre experiments were conducted at 16 °C for consistency with the intact fibre experiments. They were incubated for 25–30 min in BTS before testing inhibition because of the slow onset of suppression for these fibres.

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