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Specificity of blebbistatin, an inhibitor of myosin II

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Abstract

Blebbistatin is a small molecule inhibitor discovered in a screen for inhibitors of nonmuscle myosin IIA. We have examined the specificity and potency of the drug by assaying its effects on the actin-activated MgATPase assay of diverse members of the myosin superfamily. Blebbistatin potently inhibits several striated muscle myosins as well as vertebrate nonmuscle myosin IIA and IIB with IC_{50} values ranging from 0.5 to 5 μM. Interestingly, smooth muscle which is highly homologous to vertebrate nonmuscle myosin is only poorly inhibited (IC_{50}=80 μM). The drug potently inhibits *Dictyostelium* myosin II, but poorly inhibits *Acanthamoeba* myosin II. Blebbistatin did not inhibit representative myosin superfamily members from classes I, V, and X.

Introduction

The human genome contains about 40 myosin genes representing 12 of the 18 described classes (Berg *et al.*, 2001). In addition to their role as force transducers in muscle contraction, myosins participate in a host of cellular functions including cytokinesis, endocytosis, movement of intracellular vesicles, maintenance of stereocelia or microvillar integrity and capping of surface receptors (Sellers, 1999; Kieke and Titus, 2003). Numerous myosin isoforms are linked to familial human diseases such as deafness, cardiomyopathy, nephritis, neurological dysfunction and blood disorders (Avraham, 2003; Konhilas and Leinwand, 2003) . Most cells express numerous myosin isoforms with separate localizations and functions. Some myosins are expressed in most cell types while others are specific for specialized cells such as hair cells or photoreceptor cells. Cell permeable, high affinity, small molecule inhibitors would be very useful to study the function of individual myosins in such a complex setting, especially if inhibitors could be found that are specific for a particular myosin gene product or class. A screen of a chemical library has recently revealed an inhibitor of the nonmuscle myosin IIA (Straight *et al.*, 2003). This compound, termed blebbistatin, inhibits cytokinesis, alters the smooth movement of fish keratocytes and inhibits the spontaneous blebbing of a cell line lacking filamin. It is cell permeable and its effects are reversible upon washout of the drug.

Blebbistatin was shown to inhibit the MgATPase activity and *in vitro* motility of nonmuscle myosin IIA. At 50 μM concentration, it dramatically inhibited the actin-activated MgATPase activity of nonmuscle myosin IIB and rabbit fast skeletal muscle subfragment one (S1) (Straight *et al.*, 2003). Interestingly, it did not effectively inhibit the actin activated MgATPase activity of smooth muscle heavy meromyosin (HMM) at this concentration showing that it did not inhibit the activity of all myosin II isoforms. There was little inhibition of the activity of several other myosin class members such as myosin I, myosin V or myosin X, even at 100 μM concentration.

In the present study we have examined the dose dependency of inhibition of the actin-activated MgATPase activity of a greatly expanded pool of myosins and find that blebbistatin is an effective inhibitor of some, but not all, class II myosins. No inhibition was found of other myosin class members tested.

Experimental procedures

Preparation of proteins

Turkey gizzard smooth muscle myosin HMM was prepared as described (Sellers, 1985). Nonmuscle myosin IIA HMM (Hu *et al.*, 2001), nonmuscle myosin IIA S1 (Kovacs *et al.*, 2003), nonmuscle myosin IIB HMM (Pato *et al.*, 1996) and myosin V S1 (Wang *et al.*, 2000)

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Table 1. Inhibition constants of blebbistatin for various myosins

Myosin	Class	IC_{50} μM
Rabbit skeletal muscle	II	0.50
Porcine β-cardiac muscle	II	1.2
Scallop striated muscle	II	2.3
Human nonmuscle IIA	II	5.1
Chicken nonmuscle IIB	II	1.8
Turkey smooth muscle	II	79.6
Dictyostelium	II	4.9
Acanthamoeba	II	83
Rat myosin 1b	I	>150
Acanthamoeba myosin IC	I	>150
Mouse myosin V	V	>150
Bovine myosin X	X	>150

were expressed in baculovirus and purified as described. Myosin X HMM was engineered from a bovine myosin X clone and expressed in baculovirus (Chen *et al.*, manuscript in preparation). Phosphorylated *Acanthamoeba* myosin IC, dephosphorylated Acanthamoeba myosin II and RLC-phosphorylated *Dictyostelium* myosin II were a gift of Ed Korn (NIH), porcine β-cardiac muscle myosin S1 was a gift of Richard Moss (University of Wisconsin), rat myosin Ib was a gift of Michael Ostap (University of Pennsylvania) and scallop muscle myosin S1 was a gift of Andrew Szent-Györgyi (Brandeis University). Myosin light chain kinase (Adelstein and Klee, 1981), calmodulin (Klee, 1977) and rabbit skeletal muscle actin (Spudich and Watt, 1971) were prepared as previously described.

Actin-activated MgATPase assay

Actin-activated MgATPase activity was measured using an NADH-coupled assay in a Beckman DU 640 spectrophotometer (Wang *et al.*, 2003). Blebbistatin [a racemic mixture of the (+) and (−) enantiomers] was added from stocks dissolved in DMSO and the DMSO concentration was maintained at a constant concentration of 5% in all samples.

In Vitro Motility Assay

In vitro motility assays were performed as described in a buffer consisting of 50 mM KCl, 20 mM MOPS, 0.1 mM EGTA, 5 mM $MgCl_2$, 1 mM ATP with 50 mM DTT, 2.5 mg/mL glucose, 0.05 mg/mL glucose oxidase and 2 μg/mL catalase added to retard photobleaching (Sellers *et al.*, 1993). Actin was labeled with either rhodamine phalloidin or with a mixture of rhodamine phalloidin and Alexa-488 phalloidin as described (Sellers *et al.*, 1993). *In vitro* motility was measured using an Olympus IX70 microscope equipped for both epifluorescence and total internal reflection (TIRF) microscopy (Sakamoto *et al.*, 2003). For examination of the movement of rhodamine phalloidin-labeled F-actin, the sample was illuminated by 532 nm light and the fluorescence was observed in the epifluorescence mode.

Transient kinetic analysis of MantATP binding

The rate of ATP binding to NMIIA S1 was measured in a Kintek stopped-flow spectrofluorimeter at 25E.

Results

Blebbistatin has been shown to inhibit the enzymatic activities of HMM fragments of nonmuscle myosin IIA, nonmuscle myosin IIB and of rabbit skeletal muscle myosin S1, but not smooth muscle myosin (Straight *et al.*, 2003). We examined the dose dependency of the inhibition of the actin activated MgATPase activity of these and other myosin II isoforms. Blebbistatin potently inhibited the activity of fast rabbit skeletal muscle S1 with a half maximal inhibition at 0.5 μM concentration (Figure 1A). Two additional sarcomeric myosin II isoforms were also assayed. Porcine β-cardiac myosin S1 and scallop striated muscle S1 were inhibited with slightly higher IC_{50} values (1.2 and 2.3 μM, respectively) (Figure 1A).

The effect of blebbistatin on inhibition of the actin activated MgATPase activity of nonsarcomeric myosin II isoforms were also studied. The IC_{50} values for each of these nonmuscle myosin IIA and IIB HMM fragments were 5.1 and 1.8 μM, respectively (Figure 1B). In contrast, the actin activated MgATPase of the closely related smooth muscle HMM required considerably higher blebbistatin concentrations for inhibition (IC_{50} of about 80 μM) (Figure 1B).

Myosins from two lower eukaryotic amoebae, *Acanthamoeba castellani* and *Dictyostelium discoideum* have been particularly well studied (Brzeska and Korn, 1996). Since the actin-activated MgATPase activity of both myosins is inhibited by phosphorylation of their heavy chains, myosins with unphosphorylated heavy chains were studied (Brzeska *et al.*, 1996). The regulatory light chain of *Dictyostelium* myosin was also phosphorylated in order to obtain maximally activated myosin. Blebbistatin inhibited the actin-activated MgATPase activity of *Dictyostelium* myosin with an IC_{50} of 4.9 μM, but only poorly inhibited the activity of *Acanthamoeba* myosin (IC_{50}=83 μM) (Figure 1C).

The effect of blebbistatin on the actin-activated MgATPase activity of several unconventional (nonclass II) myosins was also measured. These include rat myosin 1b, *Acanthamoeba* myosin IC, mouse myosin Va S1, and bovine myosin X S1 (Figure 1D). No inhibition was observed with these myosins even at 100 μM blebbistatin. Similarly, no significant inhibition was detected with human myosin XV S1 (data not shown). Blebbistatin does not compete with ATP for binding to myosin as shown in Figure 2. This figure shows the time course of the increase in MantATP fluorescence that occurs when this nucleotide binds to NMIIA S1. The rate of binding is not affected by 50 μM blebbistatin.

Blebbistatin also inhibited the *in vitro* motility activity of HMM fragments of skeletal muscle myosin, NMIIA.

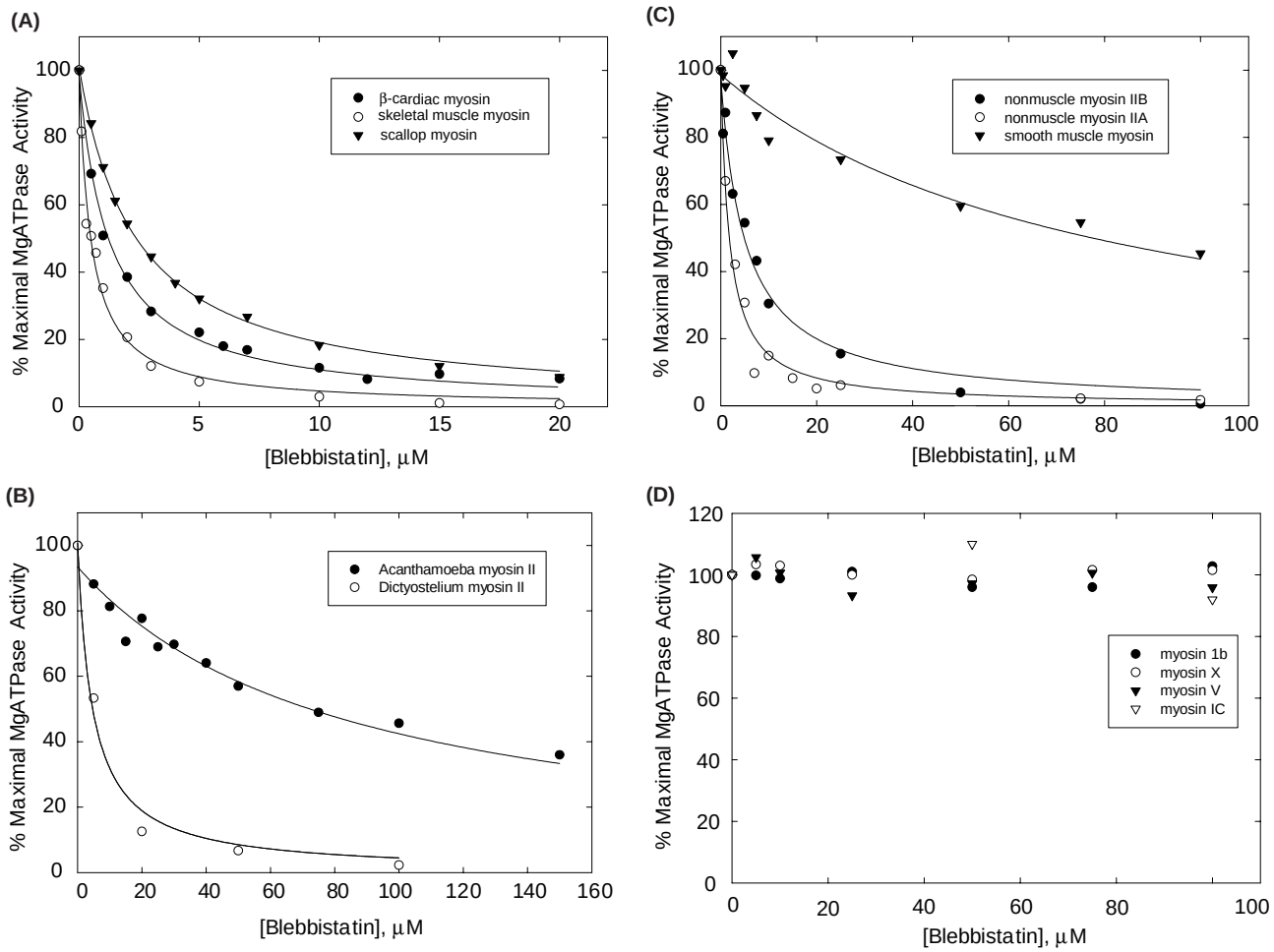

Fig. 1. Inhibition of the actin-activated MgATPase activity of various striated muscle myosins by blebbistatin. (A) Rabbit skeletal muscle S1, 100% activity=5.6/s; porcine β-cardiac muscle S1, 100% activity=2/s; scallop muscle S1, 100% activity=2.8/s. (B) Human NMIIA HMM, 100% activity=0.16/s; Chicken NMIIB HMM, 100% activity=0.32/s; turkey smooth muscle HMM, 100% activity=0.67/s; (C) *Dictyostelium myosin* II, 100% activity=1.8/s; *Acanthamoeba* myosin II, 100% activity=1.1/s (D) Rat myosin Ib, 100% activity=0.97/s; *Acanthamoeba* myosin IC, 100% activity=6.4/s; mouse mouse V S1, 100 % activity=14.4/s; bovine myosin X S1, 100% activity=5.3/s. Unless specified below, conditions were 20 mM MOPS (pH 7.0), 0.1 mM EGTA, 2 mM $MgCl_2$, 0.1 mM ATP, 10 μM actin, 200 U/mL pyruvate kinase, 40 U/mL lactate dehydrogenase, 200 μM NADH, 1 mM phophoenol pyruvate, 25°C for all assays. The assays for NMIIA HMM, NMIIB HMM and smooth muscle HMM contained 0.2 mM $CaCl_2$, 1 μM calmodulin and 2 ng/mL myosin light chain kinase. NMIIB HMM was assayed at 37°C; *Dictyostelium* myosin II and *Acanthamoeba* myosin II were assayed with 25 μM actin, 25 mM KCl, 4 mM $MgCl_2$; *Acanthamoeba* myosin IC was assayed at 3 μM actin. The data shown is from a single experiment with each myosin, but is representative of at least two independent experiments with each myosin.

Higher blebbistatin concentrations were required to fully inhibit the movement of actin filaments than were required for inhibition of the actin activated MgATPase activity (Figure 3). This suggests that the blebbistatin-inhibited state of myosin does not result in the formation of a strongly bound rigor-like molecule which would have a dominant slowing effect on the movement of the actin filament by the other, uninhibited myosin molecules. Actin filaments remain attached to the surface in the fully inhibited state, indicating that the inhibited molecules still retain the ability to interact at least weakly with actin filaments. Similar behavior is seen with unphosphorylated smooth muscle myosin that does not move actin filaments, but tethers them to the surface through interactions of the weak binding M.ADP.Pi state with actin (Cuda *et al.*, 1997). This suggests that the blebbistatin inhibited myosin probably interacts only weakly with actin and does not interfere with the ongoing movement of the remaining uninhibited myosin at intermediate concentrations.

Discussion

Blebbistatin represents an effective inhibitor of some class II myosins with IC_{50} values ranging from submicromolar to nearly 100 μM. The basis for the range of potency is not obvious from an evolutionary standpoint. Efficient inhibitions of both vertebrate and molluscan striated muscle myosins were obtained, but potent inhibition was also observed with some nonmuscle myosin II class molecules. In contrast, smooth muscle myosin II was poorly inhibited by blebbistatin. Phylogenetic analysis has shown that the heavy chain gene for smooth muscle myosin is more closely related to those of NMIIA and NMIIB than to any striated muscle myosin

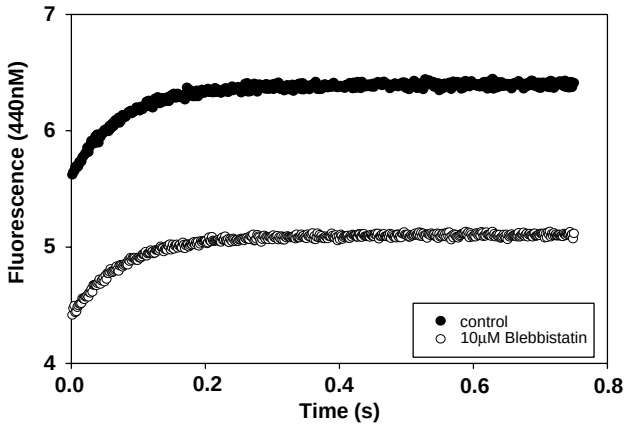

Fig. 2. Blebbistatin does not compete with ATP binding to myosin. NMIIA S1 (closed symbols) or NMIIA S1 plus blebbistatin (open symbols) was rapidly mixed with mantATP in a stopped-flow spectrofluorimeter. Excitation was at 365 nm. The final conditions were 100 mM KCl, 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 5 mM $MgCl_2$, 0.2 μM HMMIIA HMM, 2 μM ATP with or without 50 μM blebbistatin.

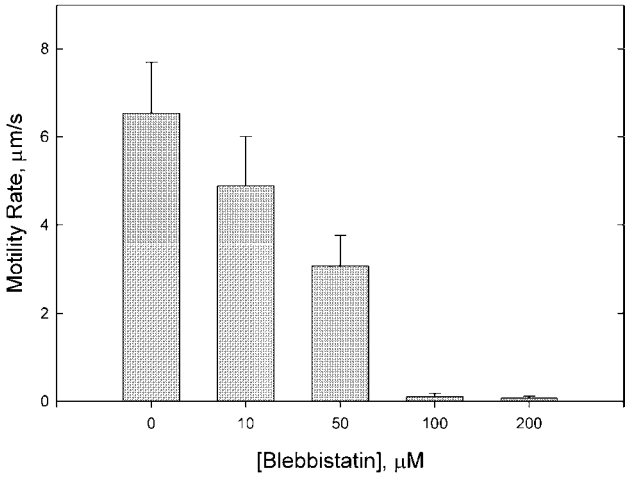

Fig. 3. Inhibition of the rate of actin filament sliding by blebbistatin. The rate of actin filament sliding over a rabbit skeletal muscle HMM-coated surface in the absence or presence of the indicated blebbistatin concentrations was measured. The data were taken within 10 s of illumination of a field. Conditions are as described in Experimental procedures.

heavy chain gene (Berg *et al.*, 2001). Vertebrate smooth muscle and nonmuscle myosin II isoforms are all regulated by phosphorylation of the regulatory light chains in contrast to vertebrate striated muscle myosins (Sellers, 1999). Therefore it is interesting that blebbistatin has only a weak inhibitory activity towards smooth muscle myosin. Smooth muscle tissue from a smooth muscle myosin knock-out mouse generates tension suggesting that nonmuscle myosin isoforms may paly some role in smooth muscle contraction (Morano *et al.*, 2000). Blebbistatin may be a useful tool to dissect the relative contributions of these two subclasses of myosin in smooth muscles.

Two other inhibitors of myosin II class molecules are available. 2,3-butanedione monoxime (BDM) is a low affinity reagent which inhibits skeletal muscle myosin II (Higuchi and Takemori, 1989), but is ineffective against several other myosins including nonmuscle myosin IIA and myosin V (Ostap, 2002) and has effects on several nonmyosin molecules (Sellin and Mcardle, 1994). A screen for small molecule inhibitors of skeletal muscle myosin II uncovered N-benzyl-p-toluene sulphonamide (BTS) as an effective inhibitor (Cheung *et al.*, 2002). This molecule inhibits rabbit skeletal muscle myosin II much more potently than other myosin II molecules, but its affects on other myosin classes have not been reported.

Blebbistatin did not inhibit the activity of myosins from any other class of myosin assayed. This should be interpreted with caution, however, as we assayed a limited number of myosins from only a few of the 18 possible classes (I, II, V, X, XV). Given the variable efficiency of inhibition of class II myosins, one should not rule out the possibility that blebbistatin might inhibit other myosins until further examination.

The results of the *in vitro* motility assays suggest that blebbistatin-inhibited myosin is trapped in a weakly bound state, which does not resist the movement of actin filaments by uninihibited myosins, but still interacts weakly with actin. This implies that to stop a cellular process driven by myosin II, it will be necessary to inhibit most of the myosin interacting with the actin filament by using blebbistatin concentrations higher than the IC_{50} values reported in this work.

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