The structural basis of blebbistatin inhibition and specificity for myosin II

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Molecular motors play a central role in cytoskeletal-mediated cellular processes and thus present an excellent target for cellular control by pharmacological agents. Yet very few such compounds have been found. We report here the structure of blebbistatin, which inhibits specific myosin isoforms, bound to the motor domain of *Dictyostelium discoideum* myosin II. This reveals the structural basis for its specificity and provides insight into the development of new agents.

Myosins are a superfamily of actin-based molecular motor proteins that use the energy of ATP hydrolysis to generate many forms of eukaryotic motility including muscle contraction, cytokinesis, intracellular trafficking and phagocytosis[1]. They are ubiquitously expressed in eukaryotic cells and can be divided into at least 18 classes based on sequence[2]. Structurally, myosins contain a highly conserved globular head domain, which consists of ATP- and actin-binding sites, a neck domain involved in light chain or calmodulin binding, and a tail domain, which anchors and positions the globular head for actin interaction.

Membrane-permeable small molecule inhibitors with high specificity for distinct myosin isoforms have great potential as tools for studying cell motility, muscle contraction and cytoskeletal diseases such as familial hypertrophic cardiomyopathy, Griscelli and Elejalde syndrome (neurological and/or immunological disorders) and deafness[3–5]. Furthermore, there is the prospect that the cytoskeleton in proliferating cancer cells can be controlled by targeting particular myosin isoforms[6]. Searches for inhibitors of class II myosins that mediate essential functions, including muscle contraction, cytokinesis, and cellular invasion and adhesion, have identified three different compounds to date. The first two, 2,3-butanedione monoxime (BDM)[7] and *N*-benzyl-*p*-toluene sulfonamide (BTS)[8], inhibit skeletal muscle myosin II. More recently, blebbistatin (**Fig. 1a**), a 1-phenyl-2-pyrrolidinone derivative, has been shown to inhibit nonmuscle myosin II and certain isoforms of muscle myosin II, but does not inhibit myosin from classes I, V and X[9]. The mechanism of inhibition by blebbistatin has been investigated by a systematic kinetic study of several myosin II isoforms and by blind docking simulations on various myosin II atomic structures[10,11]. From these studies, it has been proposed that blebbistatin behaves as an uncompetitive inhibitor and binds in the large cleft in the motor domain (50-kDa cleft), which

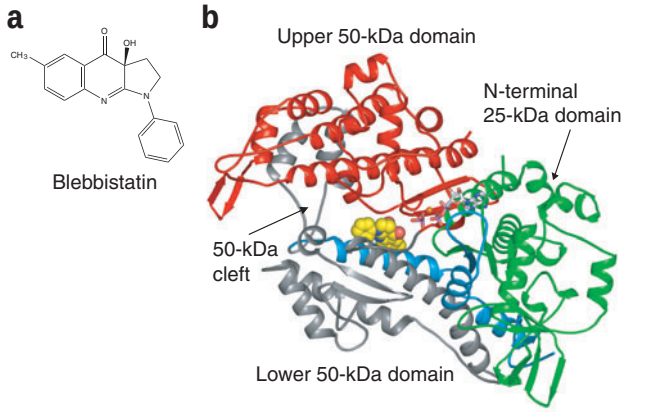

Figure 1 Blebbistatin chemical structure and binding site on MgADP–vanadate myosin II. (**a**) Chemical structure of (*S*)-blebbistatin (–)-1-phenyl-1,2,3,4-tetrahydro-4-hydroxypyrrolo[2,3-b]-7-methylquinolin-4-one. (**b**) Blebbistatin–myosin complex. Blebbistatin and MgADP–vanadate are in space-filling and ball-and-stick representations, respectively. The coordinates and X-ray data for the *D. discoideum* myosin S1dC–blebbistatin complex have been deposited in the Protein Data Bank (accession code 1YV3).

opens and closes during the contractile cycle. Blebbistatin specifically stabilizes the metastable or 'transition' state of myosin[10]. This state represents a long-lived complex of myosin with ADP and inorganic phosphate that precedes the force-generating step catalyzed by the release of phosphate when myosin rebinds to actin. At this point the 50-kDa cleft is partially closed[12]. Thus, blebbistatin inhibits the transition into force-producing states. This represents the first time that a site on myosin, other than the nucleotide- or actin-binding sites, has been implicated as the binding location of an inhibitor.

To elucidate the structural basis for blebbistatin's ATPase inhibition and its myosin specificity, we determined the structure of blebbistatin bound to the MgADP–vanadate complex of *D. discoideum* myosin II (S1dC) at a resolution of 2.0 Å (**Fig. 1b** and **Supplementary Table 1** online). This complex mimics the metastable state of myosin.

The electron density for blebbistatin is unambiguous (**Fig. 2a** and **Supplementary Fig. 1** online). It binds in a hydrophobic pocket at the apex of the 50-kDa cleft, close to the γ-phosphate-binding pocket, but is located ~20 Å away and in a radically different orientation from that predicted by the docking calculations[10]. It would seem that the inhibitor is complementary to the metastable state, consistent with the proposition that blebbistatin inhibits P_i release in the MgADP–P_i complex by stabilizing this conformation[10]. As recent structures of myosin V have indicated that P_i and MgADP release are mediated by rearrangements in nucleotide-binding site contacts (including switch I, switch II and the P loop) that coincide with complete closure

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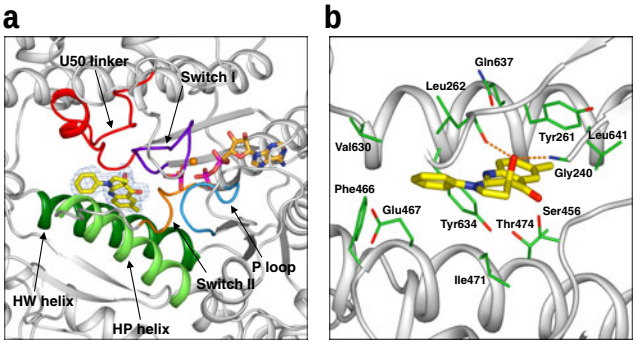

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Myosin	Selected blebbistatin contact residues				IC_{50} (μM)
Dictyostelium II	Ser456	Thr474	Tyr634	Gln637	4.9
Nonmuscle IIA	Ala	Thr	Tyr	Gln	5.1
Nonmuscle IIB	Ala	Thr	Tyr	Ser	1.8
Skeletal II	Ala	Thr	Phe	Asn	0.5
Smooth II	Ala	Thr	Tyr	Gln	79.6
Ib	Tyr	Cys	Phe	Ser	>150
Va	Tyr	Ala	Phe	Ser	>150
X	Phe	Ala	Phe	Ser	>150

Figure 2 Blebbistatin-binding site and contact residue conservation for different myosin isoforms. (**a**) Blebbistatin binds within the apex of the myosin cleft at the opposite end of the 'phosphate tube' from MgADP–vanadate. The $F_o - F_c$ electron density omit map for blebbistatin was contoured at 3 σ. (**b**) Selected amino acids interacting with blebbistatin are green and in stick representation. (**c**) Identities of residues found in nonmuscle myosin IIA and IIB, skeletal myosin II, smooth muscle myosin II, and myosin Ib, V and X corresponding to selected residues in *D. discoideum* myosin II that interact with blebbistatin are shown. The level of inhibition is shown for each myosin form[14].

of the 50-kDa cleft, it follows that blebbistatin blocks progression to this state(s) by keeping the cleft partially open. In the closed conformation of myosin V, the rigor-like state is stabilized by several specific interactions involving a highly conserved linker region (U50 linker) of the upper 50-kDa domain and the HP and HW helices of the lower 50-kDa domain[13]. In *D. discoideum* myosin II, blebbistatin sits directly between the bottom portion of this linker and the HP and HW helices within the cleft (**Fig. 2a**).

Binding seems to be controlled by the hydrophobic effect (**Fig. 2b**). The benzyl ring of blebbistatin is enclosed by the side chains of Leu262, Phe466, Glu467 and Val630, whereas the tetrahydropyrrolo ring interacts with Ser456 and Ile471. Tyr261, Thr474, Tyr634, Gln637 and Leu641 enclose the methylquinolinone component. A total of 733 $Å^2$ of protein surface area is buried by blebbistatin.

Myosin does not undergo any major conformational changes upon binding blebbistatin. Indeed, the α-carbon atoms of the inhibitor-bound and unbound MgADP–vanadate complexes superimpose with an r.m.s. deviation of 0.407 Å. However, there are significant local changes that account for the difficulty in docking blebbistatin to myosin. In particular, the side chains of Leu262 and Tyr634, which fill one corner of the apex of the 50-kDa cleft, move ~4.5 and 3 Å, respectively, to allow blebbistatin to bind (**Supplementary Fig. 2** online).

Blebbistatin is of great interest because it is specific for certain myosin isoforms. It has been shown to potently inhibit nonmuscle myosin IIB, rabbit skeletal muscle myosin II, and *D. discoideum* myosin II (**Supplementary Fig. 3** online), but does not inhibit smooth muscle myosin II, nor myosins from classes I, V or X[9–11,14]. The structural basis for this specificity can be readily explained by comparing the homologous residues to those interacting with blebbistatin in *D. discoideum* myosin II. Of these residues, Ser456, Thr474, Tyr634 and Gln637 show variability among the different myosin isoforms that is strongly correlated with their level of inhibition by blebbistatin (**Fig. 2c**). In particular, residues homologous to Ser456 in myosin I, V and X have large aromatic side chains that would prevent blebbistatin from binding. The lack of substantial inhibition of smooth muscle myosin II is, however, more difficult to explain because all of the inner sphere residues are essentially identical to skeletal muscle myosin. The structure of the ADP–AlF_4^- complex of the chicken smooth muscle motor domain is essentially identical to that of the corresponding *D. discoideum* ADP–vanadate complex. In all likelihood, the binding affinity is influenced by second sphere interactions, particularly those associated with the induced fit of blebbistatin to the 50-kDa cleft. For example, the residues associated with the new position of Leu262 are different in smooth and *D. discoideum* myosin II.

The basis of the selective activity for the (*S*)-stereoisomer of blebbistatin over the (*R*)-form is readily explained by the existence of hydrogen bonds from the chiral OH group of blebbistatin to the main chain carboxylate oxygen of Leu262 and to the main chain amide hydrogen of Gly240 (**Fig. 2b**)[9]. The alternative stereoisomer would not form an equivalent hydrogen bonding network and is thus expected to bind with lower affinity.

In conclusion, the structure of blebbistatin bound to *D. discoideum* myosin II provides a molecular model for its inhibition of myosin. Based on their structural similarities, it is likely that blebbistatin and BTS bind to the same site on myosin[15], and therefore a suite of compounds that inhibit specific myosin isoforms could be designed. The identification of new myosin-specific cleft-binding derivatives not only would provide valuable tools for dissecting complex biological processes of cell motility and muscle contraction, but also may provide useful pharmacological agents directed toward the cytoskeleton.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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