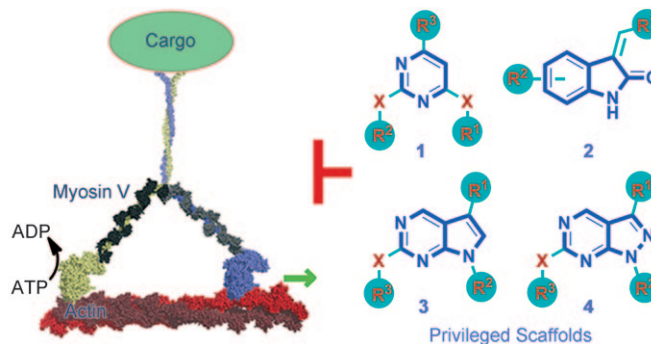# A Myosin V Inhibitor Based on Privileged Chemical Scaffolds**

Kabirul Islam, Harvey F. Chin, Adrian O. Olivares, Lauren P. Saunders, Enrique M. De La Cruz,* and Tarun M. Kapoor*

Small molecules that perturb the function of their targets on fast time scales can be powerful probes of dynamic cellular processes, such as intracellular transport.[1] A number of inhibitors for motor proteins, ATPases that drive the movement of cellular cargo, have been reported.[2] These chemical inhibitors (with micromolar potency) have served as valuable tools for the dissection of complex cellular mechanisms and have even provided an impetus for the development of chemotherapeutics that target motor proteins.[3] However, chemical inhibitors are available for only approximately 6% of the motor proteins (there are over 100 in humans[4]) involved in a variety of biological processes, including development, hearing, intracellular signaling, and muscle function.

Myosins are motor proteins that move along the actin cytoskeleton (Figure 1). Since their initial characterization as proteins that drive muscle contraction, 18 different classes of myosins have been characterized, and it is now known that myosins are involved in almost every aspect of biological motion.[5] However, specific small-molecule probes are available only for class II myosins.[2b,c] Therefore, we have set the development of chemical probes for members of the other myosin classes as our long-term goal.

The analysis of myosin structures reveals that although these enzymes bind ATP, their structures are similar to those of GTPases.[6] Good inhibitors for GTPases have been generally difficult to obtain. We reasoned that the high

Figure 1. Illustration of myosin V walking on actin filaments and structures of compounds based on "privileged" scaffolds (bold) as potential inhibitors (X = NH or CH$_2$; R$^{1-3}$ are various aliphatic or aromatic groups; ADP = adenosine diphosphate, ATP = adenosine triphosphate).

nucleotide affinity (low nanomolar), and not the structure of the nucleotide-binding pocket itself, is a key factor limiting the identification of GTPase inhibitors. This factor has also been noted in the context of inhibitor development for kinases that have unusually high ATP affinity.[7] Myosins have a K_M value for ATP that is typically in the micromolar range,[8] which raises the possibility that inhibitors for these enzymes may be more readily accessible.

To test this hypothesis, we focused on class V myosins. These motor proteins are essential for survival in eukaryotes, and mutations that impair activity give rise to pigmentation and neurological defects in mice and humans.[9] The micromechanics and mechanochemistry of myosin V have been the focus of intense research.[10] However, the precise cellular functions of myosin V remain poorly characterized, particularly in vertebrates, and a small-molecule inhibitor would be a valuable tool.

We have shown that small molecules based on "privileged" chemical scaffolds, which map to the region of chemical space occupied by known bioactive compounds, can yield diverse cellular phenotypes.[11] These results, along with other studies,[12] suggest that privileged-scaffold-based compounds may provide efficient starting points for the development of inhibitors of different target proteins. We noted that such scaffolds include pyrimidines **1**, oxindoles **2**, pyrrolopyrimidines **3**, and pyrazolopyrimidines **4** (Figure 1). We also noted that such scaffolds are common to many known kinase inhibitors.[13] The specificity of kinase inhibitors is typically examined in vitro against a large panel of known kinases. However, the ability of these inhibitors to target motor proteins has not been examined systematically. To determine whether kinase inhibitors could inhibit myosin V,

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we assembled a small collection of known kinase inhibitors, whereby we focused on various "privileged" scaffolds (Figure 1).

Myosin V exists as a multiprotein complex of over 12 polypeptides. It possesses two catalytic ATPase motor domains, called heads, which bind actin filaments and generate force (Figure 1). To test our compounds of interest, we used a recombinant protein comprising a single ATPase motor domain of chicken myosin Va. This construct, from a representative member of the myosin V class, has been extensively characterized in vitro.[14] Maximum activation of myosin ATPase requires actin polymer. By using an enzyme-coupled steady-state ATPase assay,[15] we found that two compounds, **5** and **6**, of 10 tested, reduced actin-activated enzyme activity by about 35–40% at a concentration of 100 μM (Figure 2).[16] Compound **5** is a known inhibitor of CHK1 kinase (IC_{50} = 7 nM),[13b] whereas compounds similar to **6** inhibit various cell-cycle kinases (IC_{50} values in the low-micromolar range).[13d]
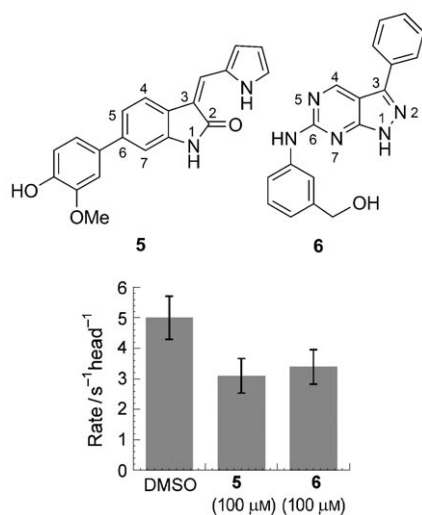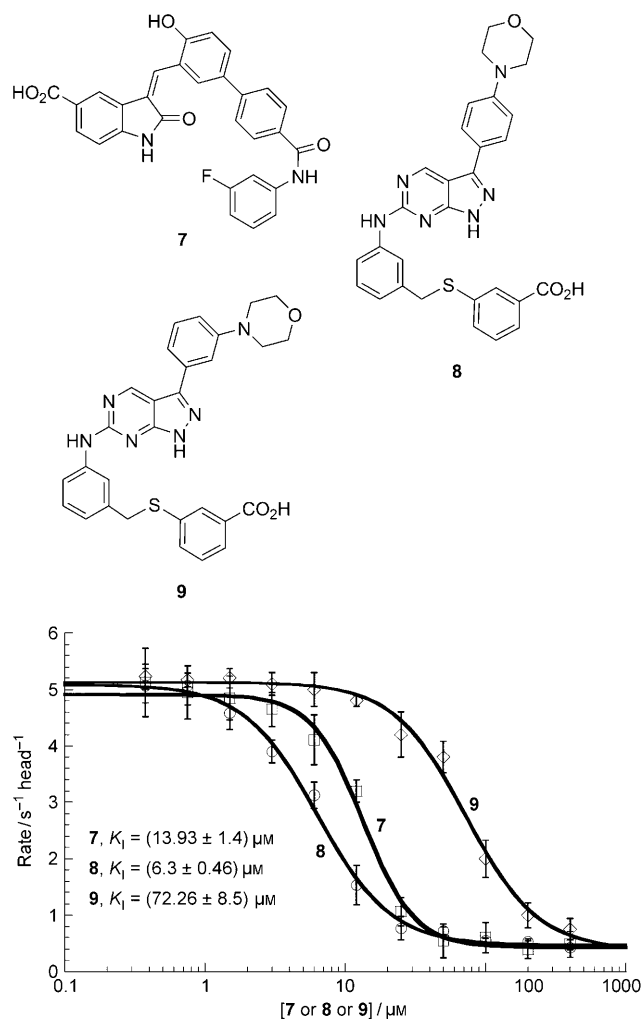

Figure 2. Initial hits **5** and **6** and their effects on the steady-state rate of actin-activated ATP hydrolysis by single-headed myosin V ($n = 3$, uncertainty bars show the standard deviation; DMSO = dimethyl sulfoxide).

Encouraged by these results, we synthesized a series of compounds based on **5** and **6** to derive inhibitors that would have decreased activity towards kinases but increased potency for myosin V.[16] To create structural diversity, we subjected commercially available oxindoles to a two-step sequence of a Knoevenagel condensation and Suzuki coupling, which could be carried out in either order.[13b] For the synthesis of pyrazolopyrimidine analogues, a four-step sequence involving a metal–halogen exchange reaction followed by hydrazine-mediated ring closure and nucleophilic aromatic substitution was adopted.[13d] A total of 60 compounds (39 oxindoles and 21 pyrazolopyrimidines) were obtained with greater than 95% purity and characterized.[16]

Testing of the oxindole-based compounds indicated that whereas substitutions at the 6-position had a modest effect on inhibitor potency, changes at the 3-position had significant

effects, whereby a biaryl moiety was most favorable (see the Supporting Information for structure–activity-relationship (SAR) data). Further modification of the biaryl moiety led to **7**, which is the most potent compound in this series ($K_{I} \approx 14$ μM; Figure 3). In the pyrazolopyrimidine series, substi-

Figure 3. Myosin V inhibitors **7**, **8**, and **9** and their dose-dependent reduction of the rate of steady-state actin-activated ATP hydrolysis (s^{-1} head^{-1}) by single-headed myosin V ($n = 3$, uncertainty bars show the standard deviation).

tution changes at the 6-position improved potency, whereby an aminobenzylthiobenzoic acid (ABTA) moiety was favored. Our SAR study (see the Supporting Information) indicated that a *meta*–*meta* substitution pattern of the ABTA moiety provides the highest potency. With this position fixed, changes at the 3-position further improved potency and led to compound **8** as the most potent myosin V inhibitor in both series ($K_{I} \approx 6$ μM; Figure 3). To the best of our knowledge, such extended aromatic substitution on oxindole and pyrazolopyrimidine ring systems has not been reported previously. Importantly, we could show that compound **8** also inhibits double-headed myosin V, an active construct that contains two catalytic domains and 12 associated light

chains,[17] with equal efficiency to that of a single-headed motor ($K_I = (5.5 \pm 2.4)$ μM).[16]

It has been noted that protein aggregation is a common mechanism of promiscuous chemical inhibitors.[18] Detergents (e.g. 0.02–0.1% Triton X) have been proposed to decrease aggregation and can be used in assays to exclude inhibitors that act through such mechanisms. The presence of 0.1% Triton X in the assay buffer led to an approximately 10-fold increase in the K_I value for compound **7**, a result consistent with the aggregation-based inhibition of myosin V.[16] In contrast, compound **8** (named myoVin-1, for myosin V inhibitor 1) showed an approximately 1.25-fold increase in the K_I value in the presence of 0.1% Triton X.[16] This sensitivity to detergent is not likely to be significant for a hydrophobic compound and potentially arises from the increase in the ATPase activity of myosin V in the presence of detergent (data not shown).

In the course of our SAR studies, we also identified compounds that are structurally similar to myoVin-1 but significantly less potent inhibitors of myosin V.[16] One such compound, **9** (Figure 3), is a positional isomer of myoVin-1 and is approximately 12 times less active ($K_I = 72 \pm 8$ μM). These results indicate that the observed inhibition of myosin V by pyrazolopyrimidine-based compounds depends on specific features of the target protein, and are not merely a consequence of changes in the physical properties of the compound (e.g. solubility).

We next examined the specificity of myoVin-1. The known SAR data for kinase inhibition by pyrazolopyrimidine-based compounds suggests that myoVin-1 should have limited activity against kinases.[13d] We tested its activity directly by using CDK1/cyclin B, a likely target for this chemical scaffold.[13d] In vitro kinase assays revealed that myoVin-1 inhibited CDK1/cyclin B with at least 30-fold lower potency ($IC_{50} > 200$ μM; Figure 4a; precise measurement was limited by the low solubility of the compound in the assay

buffer). At a 100 μM concentration, myoVin-1 did not significantly inhibit (< 5–10%) many representative kinases, including CHK1, PLK1, Abl kinase, p42 MAP kinase, casein kinase II, and Aurora kinase (Figure 4a–g). Known inhibitors of these kinases efficiently suppressed kinase activity in our assay (Figure 4a–g).[19]

We next examined the specificity of myoVin-1 towards other myosins. As representative examples in other classes, we tested skeletal-muscle myosin II and a nonmuscle myosin, myosin VI. In in vitro ATPase assays we did not observe any measurable inhibition by myoVin-1 at a concentration of 50 μM (Figure 5a,b), whereas blebbistatin, a known small-
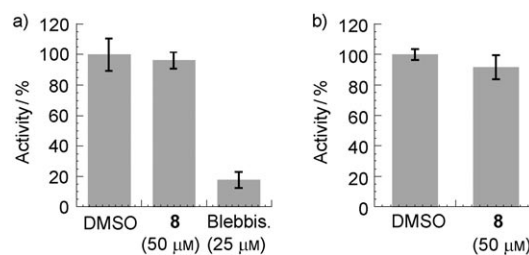
Figure 5. Analysis of the specificity of myoVin-1 (**8**) against the ATPase activities of: a) myosin II, b) myosin VI ($n = 3$, uncertainty bars show the standard deviation; Blebbis. = blebbistatin).
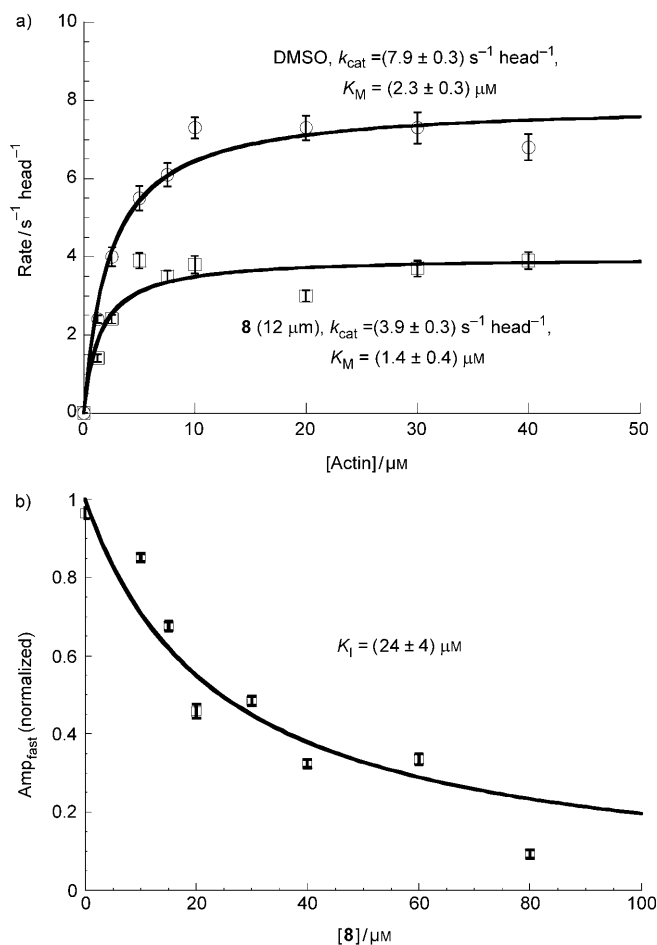
molecule inhibitor of class II myosins,[2c] significantly inhibited muscle myosin II (Figure 5a). This specificity of myoVin-1 towards myosin V is quite remarkable given that ATPase domains in members of the myosin superfamily are well-conserved.[5a]

To identify the mechanism of inhibition by myoVin-1 and identify which ATPase cycle transition(s) are targeted, we performed a series of steady-state and transient kinetic experiments. MyoVin-1 lowers the maximum turnover rate
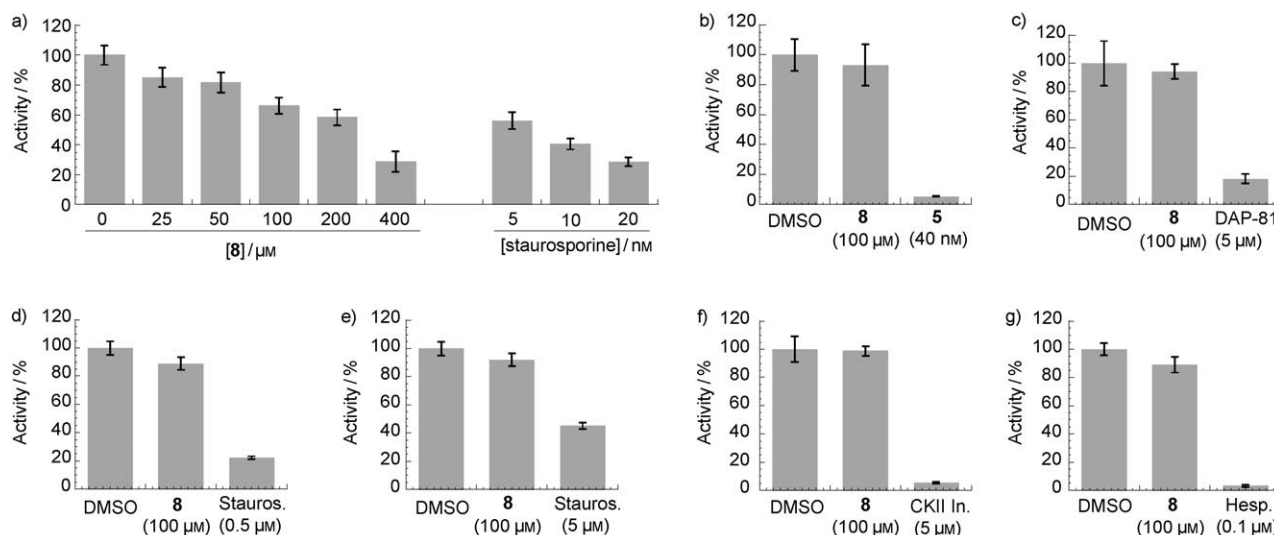
Figure 4. Analysis of the specificity of myoVin-1 (**8**) against: a) CDK1, b) CHK1, c) PLK1, d) Abl kinase, e) p42 MAP kinase, f) casein kinase II, and g) Aurora kinase ($n = 3$, uncertainty bars show the standard deviation; DAP-81 = Diaminopyrimidine-81,[11] Stauros. = staurosporine,[19a,b] CKII In. = casein kinase II inhibitor,[19c] Hesp. = hesperadin[19d]).

(k_{cat}) and apparent Michealis constant (K_M) for actin (i.e. uncompetitive inhibition is observed; Figure 6a). This effect can be explained by a decrease in rate-limiting ADP release


a) DMSO, k_{cat} = (7.9 ± 0.3) s^{-1} head^{-1},
K_M = (2.3 ± 0.3) μM

8 (12 μm), k_{cat} = (3.9 ± 0.3) s^{-1} head^{-1},
K_M = (1.4 ± 0.4) μM

b) K_I = (24 ± 4) μM

Figure 6. Steady-state and transient kinetic analysis. a) Effect of myoVin-1 (**8**; 12 μM) on the steady-state rate of ATP hydrolysis by myosin V at various concentrations of actin ($n = 3$, uncertainty bars show the standard deviation). b) Effect of myoVin-1 (**8**) on the amplitude of MANT-ADP release. Data points represent the normalized amplitudes of MANT-ADP release from actomyosin V–ADP. The solid line is the best fit to a rectangular hyperbola; uncertainty bars show the standard errors of best fits of the dissociation time course. In the absence of myoVin-1, a residual slow phase was observed as reported (Amp$_{fast}$ is the amplitude of the fast phase).[21]

from actomyosin, that is, the complex of actin and myosin V.[15,20] This hypothesis was confirmed by measuring ADP release with a fluorescent methylanthraniloyl nucleotide analogue (MANT-ATP/MANT-ADP).[21] ADP release from actomyosin–ADP prepared by equilibrating actomyosin and ADP was unaffected by myoVin-1 (data not shown). However, ADP release was inhibited by myoVin-1 if actomyosin V–ADP was prepared by mixing with ATP and allowing hydrolysis to occur. This result indicates that myoVin-1 binds to an actomyosin V intermediate populated during ATPase cycling. When ADP release was measured in this way, myoVin-1 lowered the amplitude, but not the rate constant of ADP release (Figure 6b), which indicates that myoVin-1

exchanges slowly on the timescale of ADP release (i.e. myoVin-1 binding cannot be treated as a rapid equilibrium). A myoVin-1 affinity of (24 ± 4) μM was obtained from the best fit of the dependence of the amplitudes on the concentration of myoVin-1. The slightly weaker K_I value measured with this assay in comparison to that found by steady-state kinetics is probably due to the use of a modified nucleotide analogue.[21]

These experiments indicate that myoVin-1 slows the actin-activated myosin V ATPase by specifically inhibiting ADP release from the actomyosin complex (Figure 7). Single
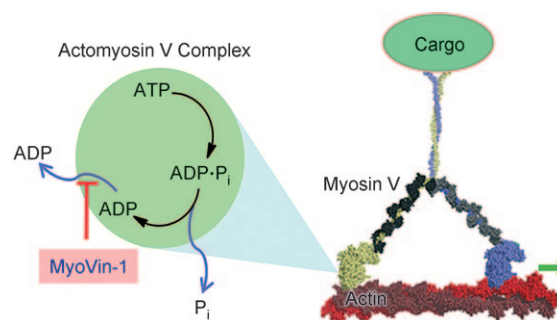
Figure 7. Mechanism of inhibition of myosin V. MyoVin-1 inhibits ADP release from the actomyosin complex.

MANT-ATP turnover measurements[22] indicated that ATP binding and the rate-limiting release of inorganic phosphate (P_i) from myosin V in the absence of actin are unaffected by myoVin-1 (data not shown), a result consistent with myoVin-1 binding to the actomyosin complex. This mechanism for the inhibition of myosins by a chemical inhibitor is unique. We anticipate that myoVin-1 will be a powerful tool for the analysis of motor-protein mechanochemistry.

In summary, from a collection of privileged chemical scaffolds, we have developed a selective myosin V inhibitor that does not compete directly with nucleotide binding. The potency of myoVin-1 is comparable to that of other known motor-protein inhibitors that are extensively used as chemical-biology probes.[2a,c] Many reported examples suggest that small changes in the chemical structure of an inhibitor can alter its mechanism of action. In particular, very minor modifications to the chemical structure of GSK923295, an inhibitor of the microtubule-based motor protein CENP-E, which, like myosin, has a GTPase-like fold, change its mechanism of inhibition from an ATP-uncompetitive to an ATP-competitive mechanism.[23] Therefore, it is possible that the initially tested pyrazolopyrimidines may be ATP-competitive, as they can be for kinases, and that the SAR-guided changes we introduced to obtain myoVin-1 altered the binding mode and inhibitory mechanism. Further structural studies will be needed to determine whether myoVin-1 binds near the nucleotide-binding pocket or at a remote site. We expect that the strategy we used to identify myoVin-1 should not only be applicable to other members of the myosin superfamily, but may be an attractive entry point into the inhibitor-discovery cycle and thus complement the high-throughput screening of large compound libraries. This

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approach is particularly useful when the protein supply is limited or when assays are complex and involve multiple components (e.g. myosins), as fewer compounds need to be tested in the primary screens. Our findings also suggest that analysis of the specificity of kinase inhibitors should not be limited to members of this superfamily, but off-target effects resulting from the inhibition of proteins with significant structural divergence, such as motor proteins, also need to be systematically tested. Although examples of inhibitor design through "scaffold hopping" have been reported previously,[24] our study provides the first example of the use of kinase inhibitors to develop compounds that target a protein with a GTPase-like fold.

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