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Review

Targeting Myosin by Blebbistatin Derivatives: Optimization and Pharmacological Potential

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Blebbistatin is a widely used inhibitor of myosin 2 that enables the study of a broad range of cytoskeleton-related processes. However, blebbistatin has several limitations hindering its applicability: it is fluorescent, poorly water soluble, cytotoxic, and prone to (photo)degradation. Despite these adverse effects, being the only available myosin 2-specific inhibitor, blebbistatin is rather a choice of necessity. Blebbistatin has been modified to improve its properties and some of the new compounds have proven to be useful replacements of the original molecule. This review summarizes recent results on blebbistatin development. We also discuss the pharmacological perspectives of these efforts, as myosins are becoming promising drug target candidates for a variety of conditions ranging from neurodegeneration to muscle disease, wound healing, and cancer metastasis.

Highlights

Blebbistatin is a myosin 2 inhibitor widely used in biochemical, cell biological, and physiological experiments investigating the roles of myosin 2 isoforms in various biological processes.

Despite its high potential in affecting myosin 2-related processes, blebbistatin has adverse features hindering its effective application in biological systems.

A variety of myosin 2-related human diseases could be targeted by a biologically safe blebbistatin derivative.

Modifications of blebbistatin's A-ring significantly reduced its inhibitory properties, suggesting that this part of the molecule is not optimal for further development.

D-ring substitutions with small, hydrophilic side chains retained the potent inhibitory properties of blebbistatin, while significantly reducing its adverse features and improving its biosafety.

Further assays on various myosin 2 isoforms with newly developed blebbistatin derivatives will be of high potential toward myosin 2 isoform-selective drug development.

Blebbistatin: A Molecular Scaffold with a Promising Future

Myosin 2 isoforms (see Glossary) play essential roles in vital processes ranging from cell division through cell motility and neuronal plasticity to contractile functions of skeletal, cardiac, and smooth muscles (Box 1) [1]. Thus, the development of myosin 2 isoform-specific lead compounds is of great potential in the treatment of various myosin- and cytoskeleton-related diseases (Table 1). To date, four allosteric inhibitory binding sites have been determined in myosin 2 (Figure 1A) including those of **blebbistatin** [2,3], pseudilins [4], the cardiac myosin-specific omecamtiv mecarbil [5], and the smooth muscle-specific CK-571 [6]. Based on structural considerations, the blebbistatin-binding site appears to be versatile and confers great potential for myosin 2 isoform-specific drug development (Figure 1B). This review focuses on blebbistatin and its recently developed derivatives.

Since its discovery and mechanistic characterization, the myosin 2 **inhibitor** blebbistatin has proven highly useful in a broad range of research areas including neuroscience, skeletal, cardiac and smooth muscle physiology, cell migration, cytokinesis, differentiation, mechanobiology, and cancer research (with over 600 PubMed citation this to date). Blebbistatin has become a standard research tool for assessing the role of tension generation and other cytoskeletal activities in practically any biological process. However, the application of blebbistatin requires extra care as the compound has several nonspecific adverse effects including cytotoxicity, phototoxicity, high fluorescence, structural instability (detailed later), which may mask possible myosin-specific effects. To overcome these hurdles, several blebbistatin derivatives have been developed [7–14]. Some of these derivatives have already been applied successfully for research purposes (e.g., in cases where a myosin 2 inhibitor with low fluorescence and high photostability was required for fluorescence imaging [15–17]). In addition to research applications, myosin 2 inhibitors based on the blebbistatin scaffold bear great potential as lead compounds for treating various myosin 2-related diseases (see below).

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Box 1. Overview of Actomyosin Structure and Blebbistatin Action

Myosins are a superfamily of eukaryotic cytoskeletal motor proteins that produce directed movement along actin filaments, powered by the free enthalpy liberated during the ATP hydrolytic cycle. The heavy-chain subunit of myosin possesses a motor (catalytic) domain containing the binding sites for actin and ATP; a neck region that acts as a force-transmitting lever binds the light chain(s) and is also a key site for regulation; and a tail region with effector functions (Figure I). Myosin-driven force production and movement are achieved via allosteric coupling between myosin's ATPase active site, the actomyosin interaction, and the force-transmitting lever during the mechanochemical cycle.

Heavy chains of Class 2 myosins dimerize through coiled-coil formation between tail regions, and dimers assemble into myosin 2 filaments, with size and dynamics showing great variation between isoforms [1]. Skeletal and cardiac muscle myosin 2 molecules, driving the contractility of voluntary and heart muscles, respectively, form stable thick filaments in muscle sarcomeres. By contrast, non-sarcomeric SMM2 and NM2 isoforms undergo reversible depolymerization regulating their activity. Mammals possess three NM2 isoforms termed NM2A, NM2B, and NM2C, with partially overlapping tissue distribution. NM2 isoforms are expressed in practically all cell types (even in muscle cells) and perform vital roles in cytokinesis, cell migration, adhesion, mechanosensing, membrane trafficking, as well as tissue and cellular morphogenesis including the outgrowth and retraction of neurites [30]. Myosin 2 isoforms show great variability in their kinetic properties, precisely tuned for their highly diverse mechanochemical functions ranging from rapid contraction of muscles to sustained tension maintenance that determines the mechanical features of tissues and organs [31].

Blebbistatin inhibits the mechanochemical cycle of myosin 2 via binding to an allosteric site in the motor domain, located between the actin and ATP-binding regions (Figure I) [2]. The inhibitor stabilizes an actin-detached state of myosin 2, and prevents the enzyme from entering force generation [20,21]. Blebbistatin inhibits nearly all myosin 2 isoforms [22,23], except for *Drosophila* and *Acanthamoeba* NM2s, with this broad-effect spectrum limiting the pharmacological applicability of the molecule. The development of blebbistatin toward isoform-selective inhibitors, as well as those with improved stability, solubility, and reduced cytotoxicity, is therefore of primary importance in its prospective clinical utilization.

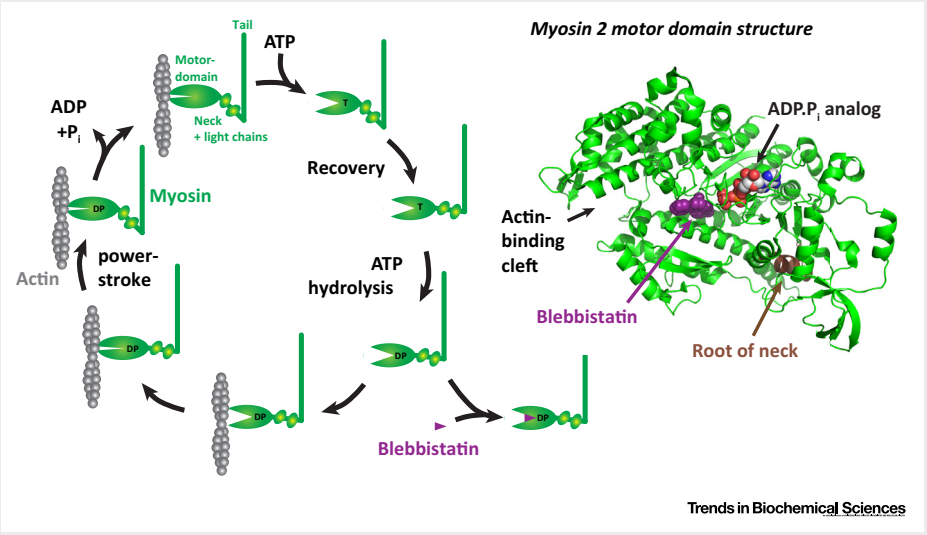

Figure I. Scheme of the Actomyosin Working Cycle (Left), and Location of the Blebbistatin-Binding Site within the Myosin Motor Domain (Right, Based on PDB Structure 1YV3). Abbreviations: T, ATP; D, ADP; P, inorganic phosphate (P_i).

Structural and Kinetic Mechanism of Blebbistatin Action

Blebbistatin was identified in 2003 as a myosin 2 inhibitor that blocks the constriction of the cleavage furrow during cytokinesis, without disruption of mitosis or contractile ring assembly [18]. Subsequent molecular mechanistic studies revealed how blebbistatin exerts its effects via interfering with the myosin 2 **mechanochemical cycle** (see Box 1 for an overview of myosin 2 structure and function and the myosin–blebbistatin interaction).

Glossary

Actin: a globular protein that forms dynamic polymer filaments. These filaments form part of the cytoskeleton and, besides binding and organizing a variety of protein complexes, serve as molecular tracks for all myosin isoforms performing a wide variety of contractile and transport functions.
ATP: a molecule hydrolyzed by many enzymes, including motor proteins, with the liberated free energy being used for metabolic processes and mechanical work.
Blebbistatin: a myosin 2-specific inhibitor that blocks the ATPase cycle of myosin 2 in an actin-detached state.
Chiral: a structure that is not superimposable by its mirror image. The activities of chiral variants (enantiomers) of a molecule may markedly differ. Blebbistatin contains a chiral carbon atom with a hydroxyl ligand, with only one enantiomer being a potent myosin 2 inhibitor.
Dimethyl sulfoxide (DMSO): a widely used colorless organic solvent that is used to dissolve substances with limited water solubility, such as blebbistatin and its derivatives. In biological experiments, DMSO concentration is generally kept below 1% to reduce solvent artifacts.
IC_{50} (half maximal inhibitory concentration) values: inhibitory constant that defines the inhibitor concentration at half-maximal effect. The IC_{50} value is indicative of, but not equivalent to, the binding affinity of the inhibitor for the enzyme.
Inhibitor: a molecule that reversibly or irreversibly reduces the rate of an enzymatic reaction.
Mechanochemical cycle: the complete sequence of enzymatic substeps of motor proteins, during which the chemical energy liberated from ATP hydrolysis is transduced into mechanical work.
Myosin: a superfamily of motor proteins found in eukaryotes. Myosins convert the energy stored in ATP into mechanical energy, in the form of translocating along their actin filament track. This movement results in processes including muscle contraction, organelle transport, cell movement, and neurite outgrowth.

Table 1. Medical Indications for Myosin 2 Inhibition

Target isoform	Medical indication		Blebbistatin effect	Refs
Non-muscle myosin 2A	Proplatelet formation			[70]
	Cancer chemotherapy	Cancer metastasis	Reduced cancer cell motility	Prospective
		Leukemia cell migration	Reduced central nervous system infiltration of leukemia cells	[51]
	Hemostasis and thrombosis	Clot retraction	Reduced platelet forces	[71]
		Arterial thrombosis	Mice: induced arterial thrombosis was ameliorated	[53,72]
	Nerve injury	Neuronal apoptosis		[73]
Non-muscle myosin 2B	Drug prevention	Prevention of relapse in methamphetamine use	Reduced context-induced drug seeking	[39]
Non-muscle myosin 2C				
NM2 (isoform not specified)	Peripheral neuropathy			Prospective
	Tumor chemotherapy	Hepatocarcinoma	Human hepatocellular carcinoma cells: antitumorigenic	[48]
		Lung carcinoma	Lung (carcinoma) cells: suppressed migration	[49]
		Benign prostatic hyperplasia		[50]
	Length-dependent neuropathy			Prospective
	Fibrosis	Lung fibrosis	Human lung fibroblasts: morphology and migration affected	[42]
		Liver fibrosis	Hepatic stellate cells: morphology and function affected	[43]
		Arthrofibrosis	Joint capsule fibroblasts: force generation and collagen production affected	[44]
			Inhibited secretion of CypA in atherosclerotic lesions	[74]
		Dermal wound healing	(Keloid) human dermal fibroblasts: migration and pathogenesis affected, reduced collagen remodeling	[45–47]
	Drug prevention	Substance (drug) use disorder	Formation and maintenance of long-term memory affected	[39–41]
	Nerve injury	Spinal cord injury	Upregulation of myosin light-chain phosphorylation in axons around the lesion site	[38]
	Periodontitis	Pathological apoptosis	Reduced receptor-mediated signaling	[75]
	Immune-related diseases	Multiple sclerosis	Reduced substrate stiffness of the endothelium	[76]
	Antiviral	Herpes virus	Limited virion entry and egress	[77]

Table 1. (continued)

Target isoform	Medical indication		Blebbistatin effect	Refs
Smooth muscle myosin 2A (tonic, vascular)	Vasodilatation	High blood pressure		[52]
		Pulmonary (arterial) hypertension	Fasudil (ROCK inhibitor): reduced pulmonary arterial pressure	[56]
		Erectile dysfunctions	Relaxation of corpus cavernosum smooth muscle	[54]
		Thrombotic disorders	Inhibited thrombus formation	[53]
Smooth muscle myosin 2B (phasic, intestinal)	Overactive bladder			[78]
	Bladder compliance			[55]
	Bladder outlet obstruction			[50]
	Erectile dysfunction		Relaxation of corpus cavernosum smooth muscle	[54]
Cardiac myosin 2β (ventricular)	Cardiomyopathies	Hypertrophic cardiomyopathy	Reduced contractility	[26]
		Dilated cardiomyopathy		[79]
Skeletal muscle myosin 2	Spasms	Nonspecific low back pain		[63]
		Spinal disk hernia, lumbago		[64]
		Stress-related occipital spasm		Prospective
		Epilepsia partialis continua		[66,67]
		Multiple sclerosis (spasticity)		[65]
		Genital dysfunctions (vaginism)		[68]
		Obstetrical muscle relaxation		Prospective
		Spasm-causing medications		Prospective
	Skeletal and cardiac myopathies	Altered ability of myofilaments to generate force	Reduced ATPase activity and reduced contractility	[60]

During the actomyosin working cycle, the binding of **ATP** to the motor domain of myosin exerts an allosteric effect leading to actomyosin dissociation. ATP is subsequently hydrolyzed in the active site of the **actin**-detached motor domain, leading to an enzyme–products complex with bound ADP and inorganic phosphate (P_i). In the uninhibited working cycle, the myosin motor domain rebinds to actin in this posthydrolytic state. During this process, the strengthening of the actomyosin interaction is coupled to a swing of myosin's force-transducing lever, leading to force generation and translocation of myosin's distal part (and the myosin 2 filament) relative to the actin filament (see Figure I in Box 1) [19]. Blebbistatin was shown to stabilize the myosin–ADP–P_i complex, thereby inhibiting the formation of strong actomyosin interactions and the entry into force-producing states (see Figure I in Box 1) [20,21]. Importantly, this effect blocks myosin in an actin-detached state, preventing artifacts that could arise from rigidified actomyosin linkages. This feature makes blebbistatin a highly useful, mildly invasive research tool in a broad range of cell biological and muscle physiological studies.

X-ray crystallographic studies revealed that blebbistatin exerts its allosteric inhibitory effect via binding into a hydrophobic pocket located at the apex of a large cleft in the actin-binding region of the myosin motor domain, which is also in the vicinity of the γ-phosphate-binding pocket of the ATPase active site (see Figure I in Box 1) [2]. Blebbistatin thus inhibits the closure of the actin-binding cleft, thereby preventing the formation of the strong actomyosin interaction.

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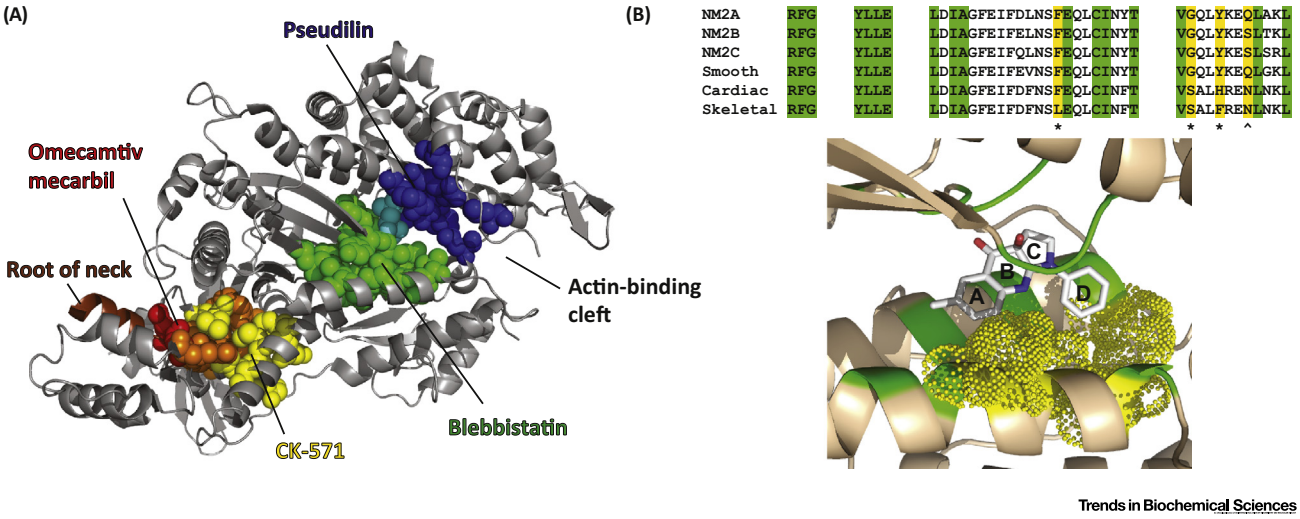

Figure 1. Myosin 2 Inhibitor Binding Sites. (A) Crystal structure of human non-muscle myosin 2C (NM2C) (PDB file 5I4E, portion comprising amino acids 1–799). Myosin inhibitor-binding sites (NM2C amino acid residues corresponding to those of target myosins within 4 Å of the bound inhibitor) are shown in space-fill representation. The blebbistatin-binding site shown (green) was obtained by placing the inhibitor into its crystallographically determined binding site (*Dictyostelium* myosin 2 structure, [2]) within a pre-relaxed NM2C crystal structure (based on PDB file 5I4E) and equilibrating for 20 ns [see also (B)]. The pseudilin-binding site (blue) was determined for *Dictyostelium* myosin 2 (PDB file 2XO8). Residues overlapping between blebbistatin- and pseudilin-binding sites are labeled in cyan. The omecamtiv mecarbil-binding site (red) was determined for human β-cardiac myosin 2 (PDB file 4PA0). This site largely overlaps (orange) with that of CK-571 (yellow) determined for chicken gizzard smooth muscle myosin (PDB file 5T45). (B) Alignment of blebbistatin contact residues [colored highlights, determined as in (A)] of six human myosin 2 isoforms. (The four sequence segments shown start at residues 234, 257, 451, and 646 in NM2A.) Residues conserved in all isoforms shown are highlighted in green, whereas yellow highlights indicate residues showing variation among isoforms. The structural image shows that the variable residues (yellow dots) that interact with the A- (labeled with ^ in the alignment) and D-rings (*) of blebbistatin provide opportunities for the development of isoform-specific inhibitors.

Blebbistatin is a **chiral** molecule, and myosin inhibition is specific to its *S* (–) enantiomer [18]. In the atomic structure of the myosin–blebbistatin complex, the hydroxyl moiety bonded to blebbistatin's chiral carbon atom forms contacts to the backbone amide group of the G240 residue and the backbone carbonyl of L262 [amino acid numbering refers to *Dictyostelium discoideum* myosin 2 (Ddm2) if not otherwise stated], and these interactions were implicated to be responsible for the striking enantioselectivity of the inhibitor [2].

Blebbistatin Sensitivity of Myosin Isoforms: Structural Basis and Enzyme Engineering

Blebbistatin inhibits a large majority of Class 2 myosin isoforms, but it does not inhibit myosins from various other classes [22,23]. The published **IC_{50} (half maximal inhibitory concentration) values** of blebbistatin reflect the highest affinity for skeletal muscle myosin 2 (submicromolar IC_{50} values) and the lowest affinity for smooth muscle myosin 2 (SMM2; 3–80 μM), with intermediate values for cardiac and non-muscle myosin 2 (NM2) isoforms (1–10 μM; Figure 2) [8–12,22–28]. Blebbistatin's specificity for myosin has been also confirmed by target fishing based on photoaffinity labeling: a photoreactive derivative of blebbistatin was photo-crosslinked to whole-cell lysates and the resulting conjugates were identified by mass spectrometry [7]. In these experiments blebbistatin was photo-crosslinked to some non-myosin proteins, too, however, with at least 10 times lower affinity.

Structural and mechanistic studies provided clues for the structural basis of the inhibitor's myosin isoform specificity and delineated routes of engineering blebbistatin sensitivity of myosin 2 isoforms via amino acid substitutions [2,23,29]. The so-called switch-2 motif of myosin's ATPase active site has the conserved amino acid sequence LDIXGFE, with the 'X' position showing class-specific variation (A or S in myosin 2; Y or F in many other myosin classes including blebbistatin-

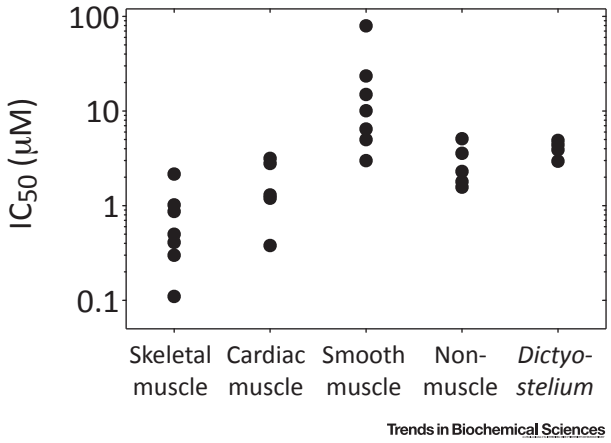

Figure 2. Inhibitory Constants (IC_{50} Values) of Blebbistatin on Myosin 2 Isoforms. Blebbistatin is most potent on skeletal muscle myosin 2, while cardiac muscle, smooth muscle and non-muscle myosin 2 isoforms (including *Dictyostelium* myosin 2) are less efficiently inhibited. Measurements were carried out using skeletal myosin isolated from rabbit fast skeletal muscles [8–12,22], cardiac myosin 2 isolated from pig heart left ventricle [22], cardiac papillary muscle from mouse [24], cardiac trabeculae from rat [25,26], smooth muscle myosin 2 isolated from turkey [22], chicken gizzard or bovine stomach [27] and chicken gizzard or rabbit uterine smooth muscle expressed in Sf9–insect cell–baculovirus system [23,28]; chicken, mouse or human non-muscle myosin 2 isoforms expressed in Sf9-baculovirus system [22,23], and *Dictyostelium* myosin 2 expressed in *Dictyostelium* expression system [8,9,22]. Abbreviation: IC_{50}, half maximal inhibitory concentration.

insensitive myosins 1, 5, and 10) [1]. S456 of Ddm2, located at this position, is one of the key blebbistatin contact residues in myosin [2]. Accordingly, the introduction of an F residue in place of the native A at this position of SMM2 and NM2 isoforms (NM2A, NM2B, NM2C) rendered these myosins blebbistatin insensitive without compromising their mechanoenzymatic activity and regulation [23]. The introduction of other bulky residues (Y, W, R, or E; but not G) at this position of NM2A also markedly decreased its blebbistatin sensitivity [23].

The 'I' position in switch 2 (I455 in Ddm2; see also above) is conserved in almost all myosins, except for *Drosophila melanogaster* non-muscle myosin 2 (DmNM2) that has an M residue at this position (M466 in DmNM2). Intriguingly, this unique variation is associated with the blebbistatin insensitivity of wild-type DmNM2, which can be sensitized by an M-to-I substitution [29]. Conversely, an I-to-M substitution at the homologous position of human NM2A rendered this isoform blebbistatin insensitive [23].

Another residue implicated in the myosin–blebbistatin interaction, T474, is also conserved in many myosin 2 isoforms, but the homologous position is occupied by C or A in blebbistatin-insensitive myosins 1b, 5a, and 10 [2]. Intriguingly, however, the T474A-equivalent substitution in SMM2 did not abolish the blebbistatin sensitivity of this isoform [23]. Similarly, substitution of S266, a residue within the blebbistatin-binding pocket that is conserved in most myosins, to an L residue did not abolish the blebbistatin sensitivity of SMM2 [23]. It is noteworthy that Y634, forming strong hydrophobic contact with blebbistatin, is at a position occupied by residues with aromatic or aliphatic side chains in most myosins (Y, H, or F in myosin 2; F, I, or L in most other classes) [1,2]. In summary, the discussed studies revealed the utility of single amino acid substitution approaches in engineering the blebbistatin sensitivity of SMM2 and NM2 isoforms.

Physicochemical Properties of Blebbistatin Relevant for Research Planning

Solubility

A well-known issue related to blebbistatin treatment is the formation of precipitates, which may interfere with imaging, block circulation, result in uncontrolled conditions, and hinder the

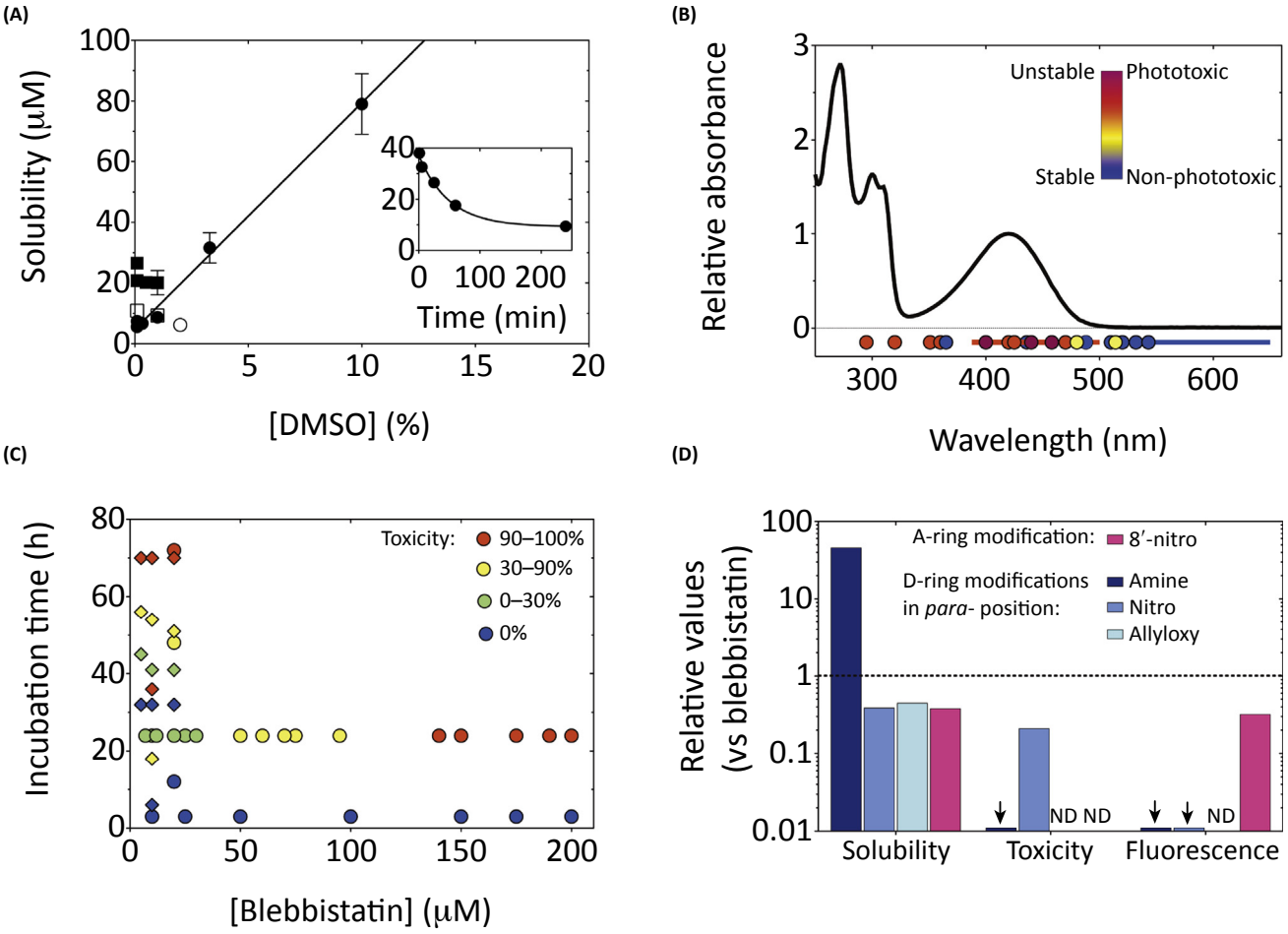

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Figure 3. Solubility and Phototoxicity of Blebbistatin, and Functional Improvements Achieved by Chemical Modifications. (A) Blebbistatin has low solubility in aqueous solutions including cell culture media, which significantly limits its applicability. Increasing DMSO concentrations improve blebbistatin solubility but can also negatively influence cell viability above 1% DMSO. Results shown were obtained in different solvents containing 0.1–10% DMSO (solid circles and squares: [7,8], open circles and squares: [9,10]). The inset shows time-dependent solubility of blebbistatin in 0.1% DMSO that follows a single exponential function with a time constant of 50 min [9] (B) The absorbance spectrum of blebbistatin (black line) shows a characteristic peak at 420 nm. Circles and colored lines indicate excitation wavelengths and wide wavelength ranges, respectively, that were used to test blebbistatin's photostability [8,11,21,33–35]. Color coding shows that blebbistatin undergoes significant photodegradation at wavelengths around its absorbance peak, associated with cytotoxicity. However, longer wavelengths have no adverse effects on blebbistatin's structure and toxicity. (C) The toxicity of blebbistatin depends on both the applied blebbistatin concentration and the incubation time in cell cultures (circles) or zebrafish embryos (diamonds) [8,9,33,34]. At short incubation times, cytotoxicity does not occur even at high blebbistatin concentrations. [Note that effective blebbistatin concentrations are uncertain above 50 μM and 1-h incubation, cf. (B).] Conversely, even low blebbistatin concentrations are highly cytotoxic at long (several days) incubation times. (D) Modifications of blebbistatin's A or D-ring with small substituents (amine [9], nitro [8,13], allyloxy [11]) were found to improve blebbistatin's adverse properties with retained or slightly reduced IC_{50} values (see also Figure 4). Notably, *para*-aminoblebbistatin showed a 46-fold increase in solubility over that of blebbistatin, with practically no fluorescence and cytotoxicity (even at long incubation times; small bars with arrows are shown only to indicate these findings) [9]. Abbreviations: DMSO, dimethyl sulfoxide; IC_{50}, half maximal inhibitory concentration; ND, not determined.

determination of effective drug concentrations [9,32]. Blebbistatin is highly hydrophobic and tends to accumulate in membranes or other lipophilic compartments such as zebrafish yolk [9] or adipose tissue in mammals [32]. These issues result from the low water solubility of blebbistatin, reported to be between 4 and 25 μM in aqueous solutions containing 0.1–2% **dimethyl sulfoxide (DMSO)** [7–10] (Figure 3A). Blebbistatin is often applied at high concentrations (>30 μM), which is above its solubility limit in aqueous solutions. Although

blebbistatin's solubility increases with DMSO concentration (Figure 3A), this is often counterproductive due to the cytotoxic effects of DMSO concentrations above 1%. Researchers therefore prefer to take advantage of blebbistatin's relatively slow precipitation kinetics that transiently allows treatments with concentration over the solubility limit (Figure 3A inset). The method for dissolving blebbistatin needs to be optimized, too, to prevent immediate precipitation, because precipitates once formed are practically undissolvable [32]. Important aspects related to blebbistatin dissolution include the use of water-free DMSO, the prevention of precipitation upon incomplete mixing of solutions, and the use of prewarmed media [32].

Photodegradation and Phototoxicity

Blebbistatin's photoinactivation, phototoxicity, and the instability of its stock solution concentration are the result of its photosensitivity that must be considered and addressed in experiments [33–35]. Studies on blebbistatin's photosensitivity, using various light sources, irradiation times, and experimental readouts, are consistent with the photosensitivity correlating with blebbistatin's absorbance spectrum, resulting in sensitivity to wavelengths below 500 nm (Figure 3B).

The precise mechanism of blebbistatin's photodegradation and phototoxicity remains elusive. Photodegradation occurs in aqueous solutions [11] and, surprisingly, the end products themselves have been demonstrated to be nontoxic to cells [33]. Instead, phototoxicity likely results from reactive oxygen species (ROS) generated during the degradation of blebbistatin [34] and/or blebbistatin binding to proteins upon illumination [33]. The illumination-independent cytotoxicity of blebbistatin (see the following discussion) suggests that degradation can also occur in the absence of light. Importantly, however, improved photostability is not necessarily accompanied by decreased phototoxicity [8]. Moreover, the cell type dependence of blebbistatin photosensitivity [34] suggests that, alongside variations in ROS sensitivity, blebbistatin degradation pathways may vary among cell lines.

Light-Independent Toxicity

Long-term incubation with blebbistatin often leads to toxic side effects even in the absence of irradiation. These myosin-independent effects have been observed in various human cancer cell lines, immortalized human fibroblasts, as well as in zebrafish embryos, with different levels of blebbistatin sensitivity in various systems [8,9,33,34]. Synthesis of published data reveals that incubation up to 1–2 days and 5–30-μM blebbistatin concentration can be generally tolerated at about 90% viability (Figure 3C). However, in short time-range experiments (incubation up to a few hours), even high concentrations of blebbistatin can be tolerated with no signs of toxicity.

Both precipitation and (photo)degradation decrease effective drug concentrations in blebbistatin solutions. It is therefore advisable to verify blebbistatin concentrations in stock solutions based on blebbistatin's absorbance peak around 425 nm (Figure 3B). Extinction coefficients were reported to be 7400 M^{-1} cm^{-1} at 422 nm [18] and 8300 M^{-1} cm^{-1} at 420 nm in DMSO (our unpublished measurements), and 6100 M^{-1} cm^{-1} at 427 nm in aqueous solution at pH 7.3 [8].

Fluorescence

Blebbistatin fluoresces in organic and aqueous solvents, and precipitated blebbistatin crystals show even enhanced fluorescence compared with the dissolved molecule [10]. Excitation and emission spectra highly depend on solvent conditions, with excitation/emission peaks reported at 410/600 nm in methanol [13], 420/560 nm in DMSO, 340/410 nm in water (at pH 7), and 435/560 nm in 1% bovine serum albumin (BSA) [32]. From a practical perspective, it is

important to note that, due to the overlap between the excitation spectra of blebbistatin, GFP, NADH, and various other fluorophores, the application of blebbistatin in cellular experiments should generally be avoided when these fluorophores are used as reporter signals.

Structure–Activity Relationship of Blebbistatin Derivatives

The blebbistatin molecule comprises a tricyclic core (termed rings A–C) linked to a phenyl group (termed ring D) via the nitrogen heteroatom of the C-ring (Figure 4, Key Figure). As mentioned earlier, the configuration around the chiral carbon with a hydroxyl ligand determines the effectiveness of blebbistatin; thus, this part of the molecule appears to be crucial for function and hence it has not been modified. Mainly due to steric reasons, other parts of the B- and the C-rings have not been substituted. However, the effects of substitutions on the A- and D-rings have been extensively characterized (Figure 4) [7–14].

Addition of ring structures to the aromatic A-ring drastically reduced the inhibitory potential of blebbistatin, and nitro or small alkyl substitutions on the A-ring did not improve inhibitory properties [12–14]. By contrast, D-ring modifications have been proven successful in ameliorating the adverse physicochemical and biological properties of blebbistatin, while its inhibitory features have largely been retained [7–11]. Large groups on the D-ring slightly decreased the inhibitory potential of blebbistatin (Figure 4A) [11]. However, small polar (e.g., hydroxyl, amino) substitutions on the D-ring significantly improved the water solubility and photostability of blebbistatin derivatives (Figure 4B–C) [9–11]. Blebbistatin's fluorescence could also be reduced or eliminated by nitro or amino substitutions in *para*- (4′-) position on the D-ring (Figure 3D) [8,9]. Moreover, the most detrimental (photo)toxic properties of the original compound could also be partially or fully eliminated by the *para*-nitro and *para*-amino substitutions, respectively (Figure 3D) [8,9]. Accordingly, the chemical stability of these blebbistatin derivatives was significantly increased (Figure 4C) [8,9,11]. Because of these positive effects, the *para*-nitro and, especially, the *para*-amino substituted versions of blebbistatin show greatly enhanced biosafety (Figure 3D). Hence, these derivatives can be used in long term and sensitive cell culture and *in vivo* experiments including functional assays elucidating the diverse cellular roles of NM2 isoforms, as well as the cytoprotective and/or direct neuroregenerative effects of NM2 inhibition.

Potential for Pharmacological Development

Non-Muscle Myosin 2

Human NM2 isoforms negatively regulate neurite outgrowth at growth cones by pulling back the outgrowing actin bundles [36]. This mechanism also reduces the motile functions of non-neuronal cells by inhibiting the formation of protrusions and reducing cellular contractility, stress fiber remodeling, focal adhesions, and tail retraction [37]. Thus, NM2 inhibition near lesion sites in spinal cord injuries or in the infarcted region after different types of stroke can lead to improved regenerative ability of the injured neuronal tissue [38]. Furthermore, NM2 inhibition by blebbistatin led to selective disruption of methamphetamine-associated memories in rats and mice [39–41]. NM2 inhibition has also been proposed to be beneficial in different types of fibrotic processes in the lung [42], liver [43], and joint capsule tissues [44], as well as in dermal wound healing [45–47] via reducing fibroblast motility and collagen production, thereby leading to reduced scar tissue formation. In addition, NM2 inhibition can be instrumental for novel forms of cancer chemotherapy by reducing cell migration of metastatic cancer cells [48–51].

Smooth Muscle Myosin 2

Different forms of smooth muscle myosin play important roles in tension maintenance of arteries and tracheae (SMM2A) and the contractility of the bladder, portal veins, and intestinal tissues

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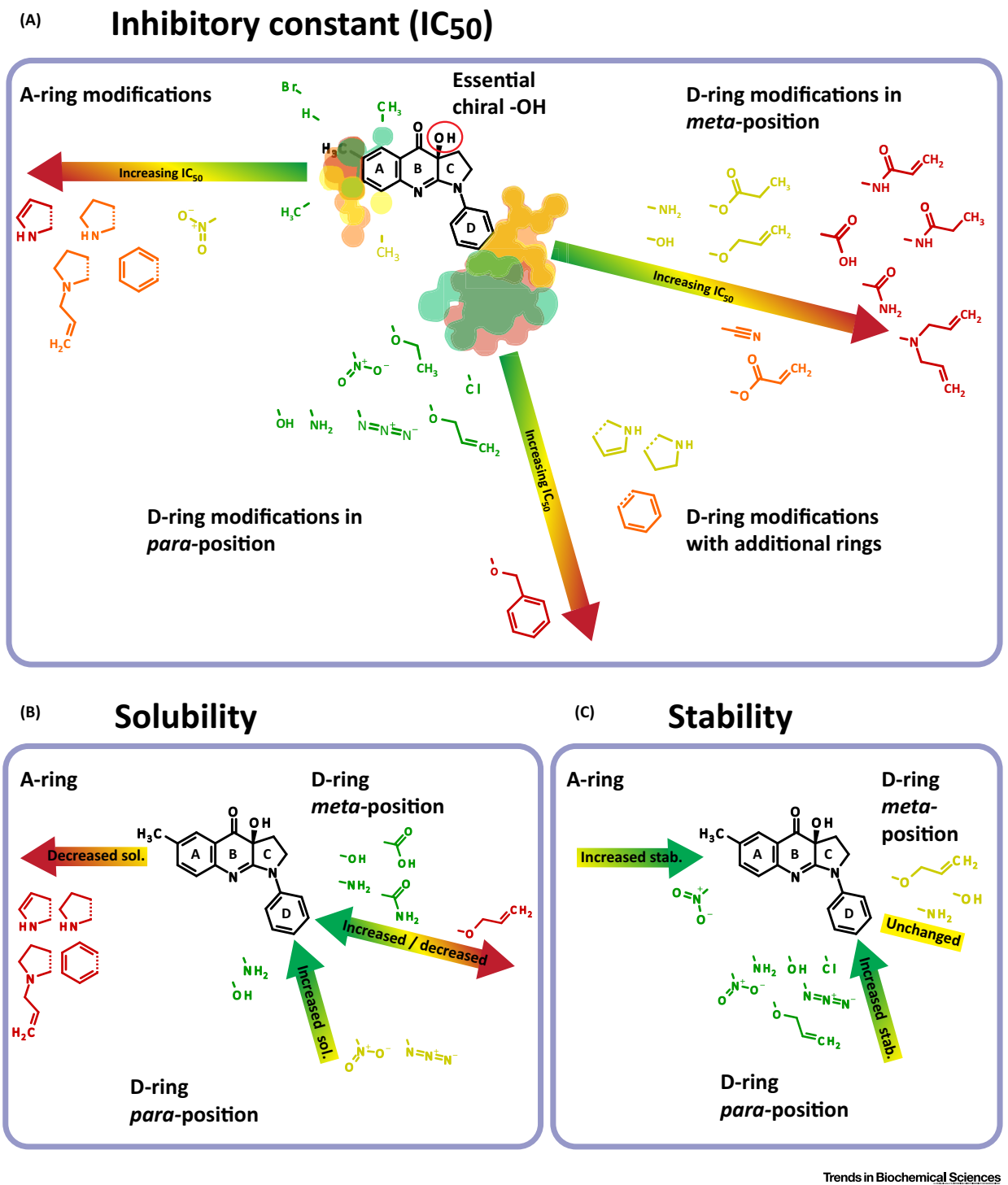
Key Figure

Effect of Blebbistatin Modifications on Inhibitory and Physicochemical Properties



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(SMM2B) [1]. Thus, the selective inhibition of these myosin forms could lead to vasodilatation that may be highly useful in the treatment of high blood pressure [52], pulmonary (arterial) hypertension, thrombotic disorders [53], and could help in overcoming erectile dysfunctions, too [54]. SMM2 inhibition has also been implicated in the treatment of overactive bladder and the maintenance of bladder compliance [50,55].

Recent pharmacological efforts to alleviate hypertension have focused on the inhibition of Rho-associated protein kinase (ROCK), an upstream regulator of SMM2 and NM2 isoforms, by fasudil [56]; and on the application of the phosphodiesterase inhibitor sildenafil (Viagra) [57]. However, the inhibition of these upstream factors influences a broad range of cellular processes. Because of the large number of its downstream effectors, ROCK inhibition affects cell cycle control, cell proliferation, cell motility, glucose uptake, and cytoskeletal remodeling processes, leading to a broad spectrum of side effects [58]. Importantly, these adverse effects could be circumvented by the development of biologically safe blebbistatin derivatives directly influencing the terminal effector myosin 2 isoforms instead of the upstream effectors.

Skeletal and Cardiac Muscle Myosin 2

Skeletal and cardiac muscles contain different types of muscle fibers of varying myosin 2 heavy-chain composition [59]. Mutations or dysfunction of these myosin isoforms can lead to different types of myopathy affecting the voluntary and heart muscles [60]. Skeletal or cardiac muscle activation through myosin (e.g., by omecamtiv mecarbil) or other protein components can be an efficient means to treat myopathies as these conditions often arise from the reduced force-producing efficiency of the relevant muscles [61,62] (Table 1). In the following section, we focus on medical indications in which the inhibition of the actomyosin system, by blocking myosin in the actin-detached (nonforce-producing) state (Box 1), could lead to temporary or permanent release of spasms and concomitant pain.

Spasms are involuntary contractions of skeletal muscles that can arise from medical treatment, genetic diseases, or psychological disorders. Skeletal muscle spasm is a characteristic symptom of nonspecific low back pain affecting the general well-being of millions of people worldwide [63]. Other modern human medical conditions that are becoming more common include spinal disk hernia and lumbago, both characterized by severe and long lasting spasm of the back muscles [64]. Spasticity is also an accompanying condition of genetic diseases such as sclerosis multiplex [65] and epilepsia partialis continua [66,67]. In addition to these diseases, the most serious symptom of some conditions of psychological origin, such as vaginismus, is the involuntary contraction of genital skeletal muscles leading to sexual discomfort and pain, difficulty in gynecological examinations, and inability to have intercourse [68]. Various medications cause skeletal muscle spasms as severe side effects, too. Thus, inhibition of myosins in the relevant striated muscles in the actin-detached form could lead to the relaxation of these muscles and relief from the accompanying pain. Blebbistatin and its derivatives can therefore become promising candidates for the treatment of such conditions, further underscoring the need for the development of specific and medically safe blebbistatin derivatives.

Figure 4. Schematic representation of the inhibitory (A), solubility (B), and stability (C) properties shows how the applied blebbistatin modifications improve (green) or worsen (red) these features [7–14]. The R(+)-enantiomer of blebbistatin (and that of its derivatives) is inactive, indicating that the chiral hydroxyl group is crucial for activity [10,18]. Considering all three important parameters shown, A-ring modifications have generally deteriorated blebbistatin's properties [12–14]. D-ring modifications in the *meta*-position have generally led to reduced inhibitory properties [10,11]. However, D-ring modifications in the *para*-position have led to significant improvements in important aspects [7–9,11]. *Para*-aminoblebbistatin, for instance, has practically retained blebbistatin's inhibitory effect, with greatly improved solubility and photostability and no cytotoxic effects (cf. Figure 3D) [9].

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Besides myosin inhibition, skeletal muscle relaxation can be achieved by targeting the neuromuscular junction using small-molecule drugs (e.g., baclofen, tizanidine, dantrolene, and benzodiazepines). However, these treatments can have severe side effects as they aspecifically affect neuronal processes in all muscle types and influence aspecific brain functions, too. Botulinum toxin (BTX), a highly potent toxin that prevents neurotransmitter release in neuromuscular junctions, has been widely used to relax muscle spasms in medical, research, and cosmetic applications. However, BTX treatment is associated with severe side effects including unintended paralysis of nontarget muscles, general muscle weakness, problems with swallowing, headache and – due to its protein nature – allergic reactions [69]. Thus, direct and isoform-specific inhibition of sarcomeric myosin 2 isoforms by blebbistatin derivatives holds promises for reducing side effects and enhancing effect profiles of novel drug candidates.

Concluding Remarks and Future Perspectives

Development of blebbistatin derivatives that retain or enhance blebbistatin's highly potent and myosin 2-specific inhibitory properties, while reducing its adverse physicochemical and toxic features, is of urgent need. Novel, biologically safe, potent, and possibly myosin isoform-specific derivatives could be used to effectively treat a wide range of medical conditions affecting the quality of life of millions of people worldwide (Table 1). Moreover, the direct inhibition of myosin 2 isoforms instead of that of their upstream regulators (e.g., ROCK) could significantly reduce the side effect profile of future drug candidates in various medical indications. To explore the possibilities for isoform-specific inhibitors, effect profiles of blebbistatin derivatives must be assessed for a broad range of skeletal, cardiac, smooth muscle, and non-muscle myosin 2 isoforms. Individual or combined modifications of blebbistatin's A- and D-rings and, possibly, that of the C-ring, may lead to more effective and/or selective candidates for drug development (see Outstanding Questions).

Outstanding Questions

Can blebbistatin's physicochemical properties, inhibitory activity, and biosafety be further improved by A-, D-, and potentially, C-ring modifications, or combinations thereof?

What are the prospects for the development of a myosin 2 isoform-specific blebbistatin derivative in selectively and efficiently treating various myosin 2-related diseases?

Can the direct inhibition of myosin 2 be used to circumvent the side effects of inhibitors of upstream processes?

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Disclaimer Statement

A.M-C. is an employee of Printnet Ltd., owner of a patent on *para*-aminoblebbistatin and its derivatives (PCT/EP2017/051829). A.M-C. is an owner, A.Á.R. is an employee of Optopharma Ltd., an enterprise distributing blebbistatin derivatives.

Supplemental Information

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