

SELECTIVE CHEMICAL INTERVENTION IN BIOLOGICAL SYSTEMS: THE SMALL MOLECULE TOOL, (*S*)-(-)-BLEBBISTATIN

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Received: 17th January 2005 / Published: 22nd July 2005

ABSTRACT

Selective small molecule inhibitors of protein function provide a method of studying biological processes that is often complementary to classical genetic and RNAi-based approaches. This article focuses on a recently identified small molecule known as blebbistatin. We review blebbistatin's discovery, biological characterization, selectivity and continuing use. Synthetic chemistry has played a key role in the blebbistatin story and we also review our recent work relating to the asymmetric synthesis and absolute stereochemical assignment of the active enantiomer, (*S*)-(-)-blebbistatin. High-throughput synthetic approaches to blebbistatin analogues are discussed and a novel analogue is described that has significantly improved physical properties for use in fluorescence-based imaging experiments on live cells. This article looks to emphasize the multidisciplinary nature of research projects in chemical genetics.

INTRODUCTION

The search for novel small molecule modulators of protein function continues to gather momentum in academia. For example, the NIH roadmaps in "accelerating medical discovery", "building blocks, biological pathways and networks" and "molecular libraries and imaging" provide a medium- to long-term view of the US commitment to research in chemical genetics (defined as a discovery platform using small molecules that alter the function of specific proteins in place of mutations).

This revival in the use of small molecules in academic biology has occurred because they provide a method of dissecting biological processes that is often complementary to classical genetics and RNAi technology. Interestingly, it seems that there is even a place for small molecules in the emerging field of "systems biology". The World Technology Evaluation Centre (WTEC) in its list of the technologies required for systems biology research includes 'tools for analyzing the spatial and temporal behaviour of networks'. Two recent review articles support this view [1,2]. The ability to reversibly control the activity of a protein as a function of time and location is a key advantage of small molecules. This is achieved by "washing in" or "out" the small molecule at a chosen time in a chosen cell type - both factors that are under the researcher's control. "Caging" the small molecule can also provide the required level of control [3]. Figure 1 lists several other reasons why novel small molecules are of importance in post-genomic science.

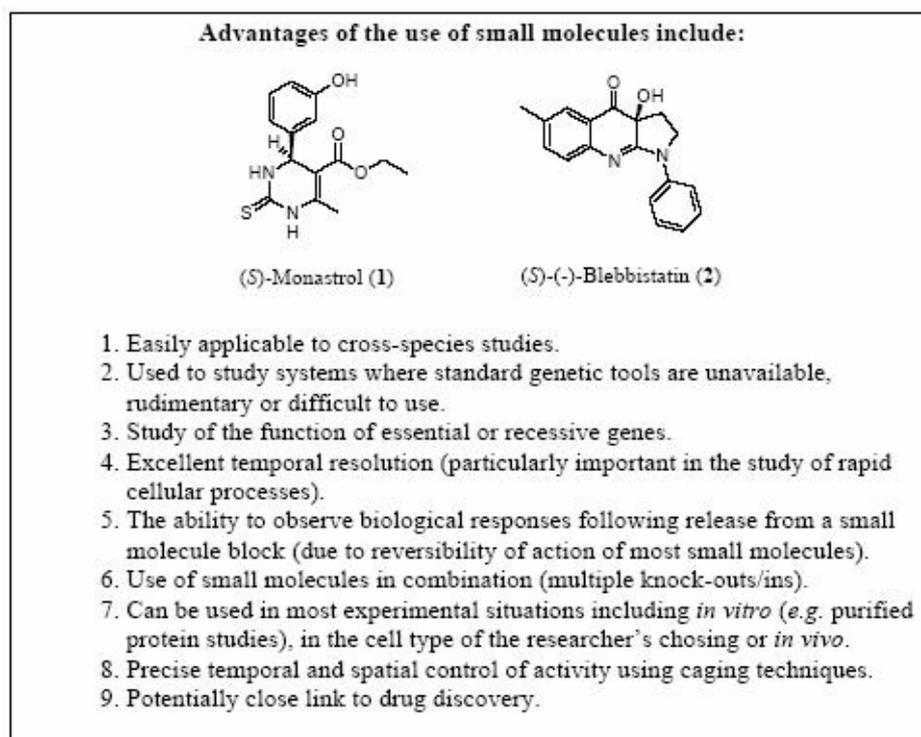


Figure 1. Small molecules and chemical genetics: *Forward chemical genetics* involves identifying a phenotype in an organism or cell caused by a small molecule and then identifying the protein target (c.f. forward genetics); *Reverse chemical genetics* involves selection of a protein target of interest and screening for small molecules that affect the protein's activity (c.f. reverse genetics). All definitions relating to chemical genetics are taken from <http://iccb.med.harvard.edu>.

The recently identified small molecule tool monastrol (**1**) provides an excellent example of the impact that a selective modulator of protein function can have [4,5].

In this article, we review the literature surrounding another example that is having an equally dramatic influence on both the biology and chemistry communities. Since its first report in March 2003 [6], 22 peer-reviewed research papers (as of 4th April 2005) have been published focusing on the use of the small molecule tool, (*S*)-(-)-blebbistatin (**2**).

IDENTIFICATION OF (±)-BLEBBISTATIN BY HIGH-THROUGHPUT SCREENING

Small molecules that modulate the activity of cytoskeletal proteins are of great use to cell biologists [7]. For example latrunculin A, a marine natural product that binds monomeric actin [8] and prevents its incorporation into filaments, has been used extensively to study the cellular role of actin (for a review see [7]). However, there are many areas of cytoskeletal research that lack the necessary small molecule tools. For example, the detailed study of cytokinesis in mammalian cells would benefit from a small molecule that is capable of preventing ingression of the cleavage furrow *without* preventing furrow assembly. Halting the cell cycle in cytokinesis can be achieved by inhibiting the action of *non-muscle* myosin II (NMII) [9]. Unfortunately the widely used (and controversial [10]) *muscle* myosin II inhibitor, BDM [11], has been reported not to inhibit NMII [12]. Therefore, in order to study cytokinesis in more detail using a chemical genetic approach it was necessary to *discover* a novel small molecule that inhibits the ATPase activity of NMII.

Mitchison and co-workers employed high-throughput screening (HTS) of commercially available chemical libraries to identify inhibitors of NMIIA ATPase activity [6], a strategy that had already been successfully used in their laboratory to discover novel inhibitors of muscle myosin II [12]. From over 16,000 screening compounds, 4 inhibitors of NMIIA ATPase activity were identified (Fig. 2, panel B), of which (±)-blebbistatin was the most potent.

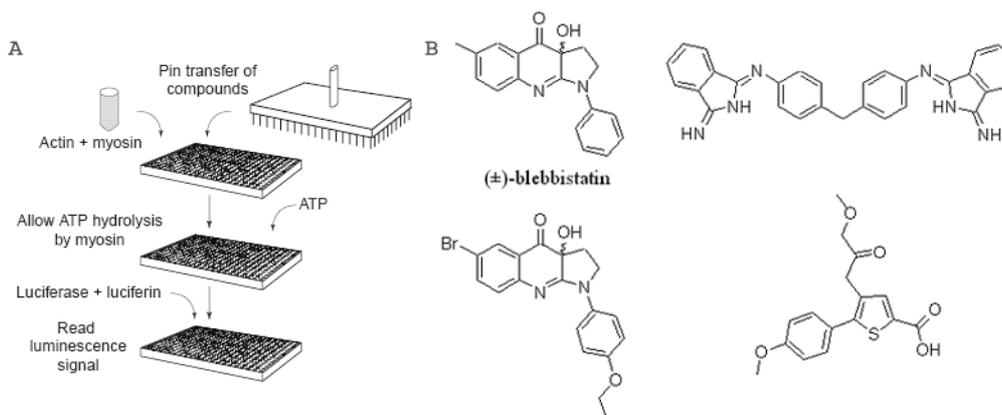


Figure 2. A reverse chemical genetic approach to discover novel small molecule tools: **A)** Diagrammatic representation of the coupled assay procedure used to identify inhibitors of non-muscle myosin IIA (one of three isoforms that exist). The assay was performed by adding DMSO solutions of small molecules to wells containing a solution of actin and human platelet NMIIA. The assay was performed in a 1536 well format, with nL volumes of solutions being transferred using an automated pin transfer device. The assay was then initiated by the addition of ATP. The assay plates were incubated to allow ATP hydrolysis to occur prior to the addition of a solution of luciferase and luciferin. The resulting luminescence was then recorded as a direct measure of the inhibitory effect of each small molecule; the greater the luminescence the more effective an inhibitor the small molecule was. **B)** The four inhibitors of NMIIA identified by HTS including (±)-blebbistatin [6]. Copyright Nature Publishing Group, <http://cellbio.nature.com> [12].

PRELIMINARY BIOLOGICAL CHARACTERIZATION OF (±)-BLEBBISTATIN

As with all reverse chemical genetic approaches, the next stage of this programme was to determine the effect of (±)-blebbistatin upon NMII-dependent processes in cells. (±)-Blebbistatin at a concentration of 100 nM was found to inhibit cell **blebbing** in M2 cells (explaining the compound's name) and to perturb cell motility in vertebrate cells (goldfish keratocyte assay) [6]. Consistent with the goals of the study, 100 nM (±)-blebbistatin was also found to block cleavage furrow contraction in dividing *Xenopus* tissue culture (XTC) cells [6]. Importantly correct assembly of the cleavage furrow occurred in the presence of (±)-blebbistatin, with the localization of non-muscle myosin II, anillin (another component of the cleavage furrow [13]) and microtubules being unaffected. All of the inhibitory effects of (±)-blebbistatin were found to be reversible, the cells re-entering the cell cycle after 'washing out' of the inhibitor.

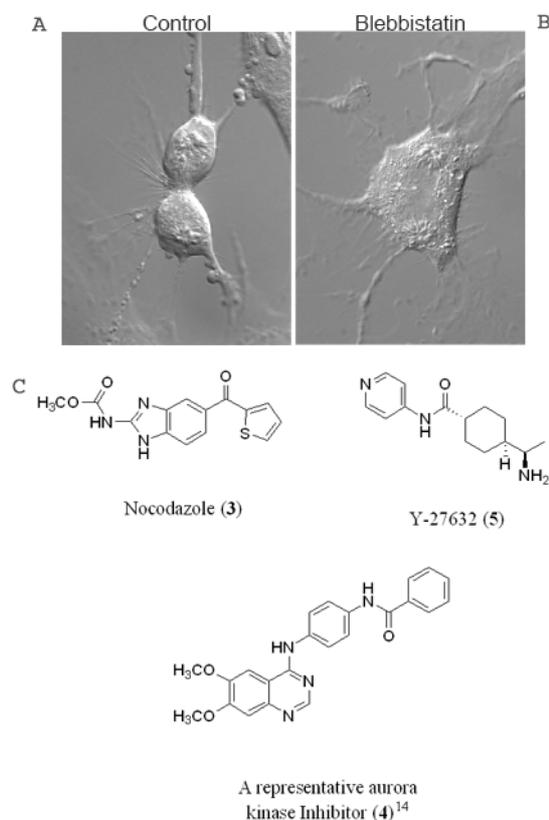


Figure 3. The effect of (\pm)-blebbistatin on cytokinesis. **Image A** shows an XTC cell undergoing normal cell division in the absence of drug treatment (control cell), note the presence of the contracted cleavage furrow. **Image B** shows the same cell type treated with 100 mM (\pm)-blebbistatin, Note the complete absence of cleavage furrow contraction despite successful chromosome segregation. Movies showing this process in more detail are also available in the supplementary material for [6]. (<http://www.sciencemag.org/cgi/content/full/299/5613/1743/DC1>). **C**) The structures of nocodazole (3), aurora kinase inhibitor (4) and Y-27634 (5). These small molecules were used in conjunction with (\pm)-blebbistatin to carry out double chemical knockout experiments. Copyright Science Magazine.

The ability of (\pm)-blebbistatin to block furrow cleavage without affecting the localization of other cytoskeletal components allowed 'double chemical knockout experiments' to be carried out. This involved incubating cells in the presence of both (\pm)-blebbistatin and other small molecule tools. For example, incubation of (\pm)-blebbistatin-arrested HeLa cells with nocodazole (3) (a small molecule tubulin depolymerizer) resulted in cells with delocalized non-muscle myosin II (NMII) and anillin and with no microtubule mid-zone, thus demonstrating an essential role for microtubules in maintaining furrow component localization. An analogous experiment in which nocodazole (3) was replaced by an aurora kinase inhibitor (e.g. 4) resulted in the delocalization of NMII *but not anillin*. The mid-zone microtubules were also disrupted. This result provided the first evidence that the localization of these two furrow components is *independently regulated*.

A further experiment involving treatment of (\pm)-blebbistatin-arrested cells with the rho-kinase inhibitor, Y-27634 (**5**), also led to delocalization of NMII and not anillin, but this time had no effect upon the mid-zone microtubules.

It appears therefore that the action of rho-kinase is required for localization of NMII into the cleavage furrow and that aurora kinase is required for mid-zone microtubule organization. Following this initial report on the use of (\pm)-blebbistatin, several other groups have used this small molecule tool to study other NMII-dependent cellular process. This literature is briefly reviewed in the section entitled (*S*)-(-)-Blebbistatin (**2**) as a Molecular Tool.

THE CHEMICAL STRUCTURE OF (\pm)-BLEBBISTATIN

A key step in HTS approaches using commercially available small molecule collections is to confirm the structure of the initially identified hit. In the case of (\pm)-blebbistatin, this process proved particularly interesting as (\pm)-blebbistatin itself was not present in the commercial collection that was used. In fact, the compound present in the "(\pm)-blebbistatin hit well" was listed as **6** (Fig. 4). A repurchased and freshly dissolved sample of **6** did not inhibit NMII.

The relatively rapid conversion of a DMSO stock of **6** from a colourless to bright yellow solution suggested, however, that **6** degrades to produce the NMII inhibitor. Whilst not the first time that a degradation product or contaminant of the intended inhibitor has proved to be the bioactive component [15], these cases are relatively rare. Initial studies carried out in the Mitchison laboratory, showed that an "aged" DMSO solution of **6** does indeed degrade to (\pm)-blebbistatin (Fig. 4).

The 'degradation' of **6** to (\pm)-blebbistatin could occur via quinolone **7** followed by reaction with singlet oxygen, analogous to our synthetic route [16]. Evidence in support of this hypothesis could be obtained by conducting experiments with [$^{18}\text{O}_2$]-oxygen gas and these studies are ongoing in our laboratory. Subsequent studies from the Mitchison and Westwood laboratories have provided a more efficient route to (\pm)-blebbistatin via quinolone **7** [16].

Selective Chemical Invention in Biological Systems

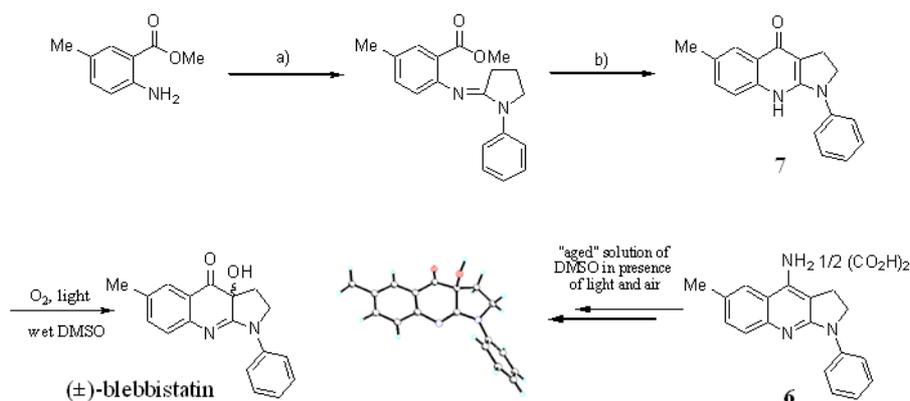
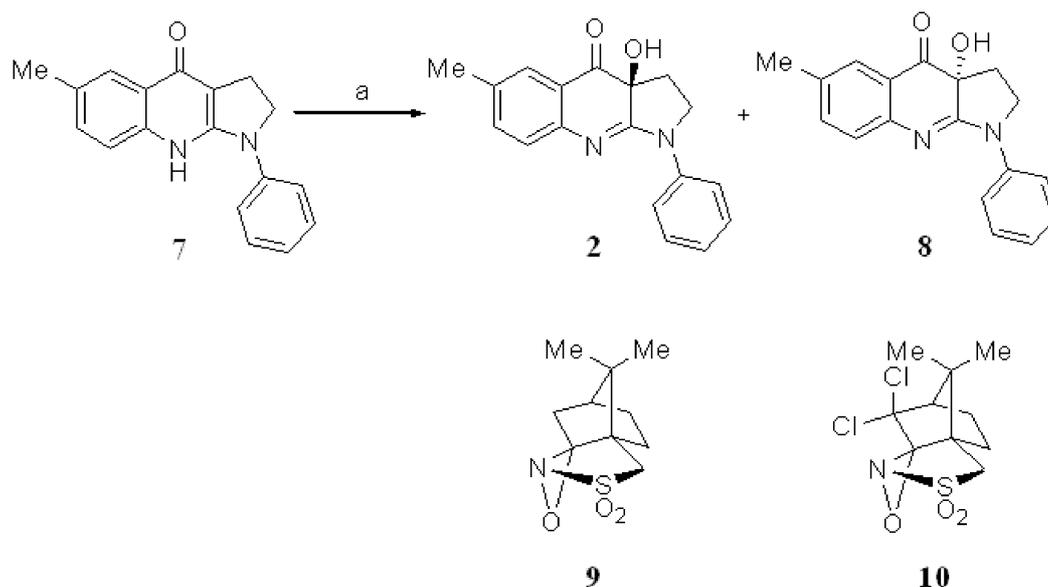


Figure 4. Preparation of (±)-blebbistatin. Reagents and conditions: a) i) POCl_3 , CH_2Cl_2 , 25°C , 3 h; ii) *N*-phenyl-2-pyrrolidinone, 40°C , 16 h, 41%; b) LiHMDS (3 equiv), -78°C to 0°C , 3 h, 90%; c) O_2 , hv, rose Bengal, DMSO, 25°C , 3 h, 29%; or O_2 , hv, rose Bengal, 25°C , 3 h, THF, 26%. LiHMDS = lithium bis(trimethylsilyl)amide [16]. X-ray crystallographic analysis of a sample of (±)-blebbistatin confirmed the atom connectivity is as shown.

(±)-Blebbistatin possesses a single chiral centre. It is well established that one enantiomer of a bioactive compound is often more potent than the other, or even that one enantiomer is inactive or induces a different unrelated phenotype. Therefore, Mitchison and co-workers separated the enantiomers of (±)-blebbistatin using chiral HPLC techniques and subjected them to individual biological testing [6]. *In vitro* assays with (-)-blebbistatin (**2**) (the enantiomer that rotates the plane of plane polarized light in the -ve direction) gave an IC_{50} value of ~ 2 mM against NMIIA, whilst (+)-blebbistatin (**8**) (Fig. 5) was inactive. Cellular assays performed with (-)-**2** or (+)-blebbistatin (**8**) demonstrated that only (-)-blebbistatin (**2**) was able to arrest cells in cytokinesis, consistent with the trend established *in vitro*. Whilst a report [17] has called into question these results, it is now clear that the discrepancies arise from the lower levels of optical purity associated with some commercially available sources of **2** and **8**. In the light of these results, it became desirable to develop a highly efficient route to optically pure samples of both **2** and **8**.

The selective formation of chiral centres presents a significant challenge to the organic chemist. However, precedent exists for the use of *N*-sulfonyloxaziridines such as **9** and **10** (Fig. 5) to perform asymmetric hydroxylations of ketone enolates in high yield and with excellent enantiomeric excesses (e.e., the percentage difference in the abundance of 2 enantiomers formed in a reaction e.g. if the enantiomer ratio was 95:5 the e.e. would be 90%) [18,19]. It was envisaged that optically enriched (-)-**2** or (+)-blebbistatin (**8**) could be prepared from quinolone **7** using this chemical methodology (Fig. 5) [16].

This late stage oxidation was attractive as both enantiomers could be prepared in a single step from a common intermediate. Treatment of the lithium enolate of quinolone **7** with oxaziridine **9** gave optically enriched (-)-blebbistatin (**2**) in moderate e.e. (entry 2, Fig. 5). The reaction temperature, oxaziridine, and base were all varied in order to identify optimal conditions for the formation of (-)-blebbistatin (**2**). These studies resulted in a procedure that enables the synthesis of highly optically enriched (>99.5% e.e.) **2** after a single recrystallization step (entry 4).



ENTRY	DAVIS OXAZIRIDINE	TEMPERATURE (°C) ^a	BASE	TIME(H)	% YIELD	% E.E. OF 2 ^b
1	9	-78	LiHMDS	16	0	N/A
2	9	-10	LiHMDS	16	70	42
3	10	-78	LiHMDS	16	0	N/A
4	10	-10	LiHMDS	16	82	83
5	10	-78	NaHMDS	16	69	82
6	9	0	LDA	16	90	20

Figure 5. Optimization of asymmetric hydroxylation of **7** to give enantiomerically enriched (-)-(**2**) using the Davis oxaziridine methodology [16]. The hydroxylation reaction was performed using different bases; LiHMDS (lithium bis-(trimethylsilyl)amide), NaHMDS (sodium bis-(trimethylsilyl)amide) and LDA (lithium di-isopropylamide). Various reaction temperatures and two commercially available oxaziridines were also tested in order to determine the optimal reaction conditions. The e.e. values were determined using chiral HPLC analysis of the crude reaction mixture.

Neurological and cancer biology studies using (±)-blebbistatin have identified myosin II as a putative drug target (see the section entitled (*S*)-(-)-Blebbistatin (**2**) as a Molecular Tool) [20,21]. As a result (-)-blebbistatin (**2**) could be considered as a lead compound for pharmaceutical development.

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An efficient route to highly optically enriched (-)-blebbistatin (**2**) and its analogues is therefore of additional significance given the high level of optical purity typically required in even potential therapeutics. Preliminary studies from our group indicate that whilst subtle variations in e.e. do occur, this methodology is very tolerant of substitutions in either aromatic ring in **7**.

In addition to being able to synthesize small molecule tools in the required purity levels, it is also necessary to determine their absolute stereochemistry (where relevant). Determining if the bioactive enantiomer is *R* or *S* aids computer modelling-based studies designed to identify (-)-blebbistatin's (**2**) myosin binding site [17]. Knowing the absolute stereochemistry is also essential in the planning of alternative synthetic routes to (-)-blebbistatin (**2**) and its analogues. As (-)-blebbistatin (**2**) contains no "heavy atoms", it is difficult to determine its absolute stereochemistry using X-ray diffraction techniques in the absence of extremely high quality crystals. In order to overcome this problem, a heavy atom (bromine)-containing analogue **11** was prepared (Fig. 6). X-ray crystallographic analysis of **11** showed that its absolute stereochemistry was *S*. Subsequent replacement of the bromine in (*S*)-(-)-**11** with a hydrogen atom using catalytic hydrogenation gave exclusively (-)-blebbistatin (**2**) (confirmed by comparison with authentic material using chiral HPLC). Therefore, *the absolute stereochemistry of (-)-blebbistatin (2) is S*. Further evidence in support of this conclusion comes from a recently published X-ray crystal structure of (*S*)-(-)-blebbistatin (**2**) bound to the motor domain of *Dictyostelium discoideum* myosin II (see section entitled Future Studies).

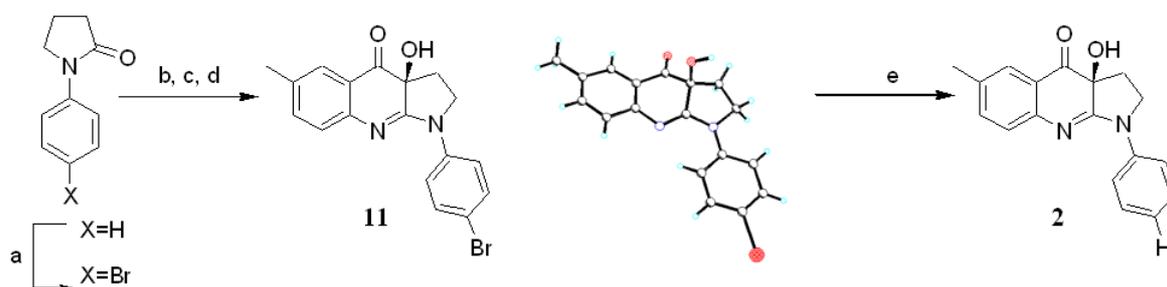


Figure 6. Assignment of the absolute stereochemistry of (*S*)-(-)-blebbistatin (**2**). Reagents and conditions: a) NBS, DMF, 25°C, 2 days, 50%; b) i) POCl₃, CH₂Cl₂, 25°C, 3 h; ii) methyl 5-methylanthranilate, 40°C, 16 h, 26%; c) LiHMDS (3 equiv), -78°C to 0°C, 3 h, 60%; d) i) LiHMDS (1.2 equiv), THF, -78°C; ii) **10** (2.4 equiv), -10°C, 16 h, 68%, 88% *ee*; recrystallization from acetonitrile >99% e.e., [α]_D₂₆ -526 (c=0.1, CH₂Cl₂); f) NBS = *N*-bromosuccinimide, DMF = *N,N*-dimethylformamide.

ASSESSING THE SELECTIVITY OF SMALL MOLECULE TOOLS

The most frequent criticism of the use of small molecules to study biological processes is that they lack sufficient selectivity to enable "clean" biological questions to be asked. With the increase in methods [22] of assessing selectivity, the onus is increasingly on the developer of novel small molecule tools to demonstrate just how selective the latest addition to the toolbox is. In this case, a large superfamily of myosins that function as actin-dependent motor proteins is known to exist. Each family member possesses a globular motor domain [23]. A recent phylogenetic analysis using the motor domain sequences of known or predicted myosins from several species divided the superfamily into 18 classes [24]. Additionally, some myosin classes are further divided into subclasses [25]. For example the myosin II class is partitioned into subclasses that include skeletal, cardiac, smooth muscle and non-muscle. Each subclass may contain numerous isoforms (for example nonmuscle myosin II exists as three isoforms A, B and C) [26]. Assessing selectivity is a daunting (but essential) task even when it is assumed that no other protein families are being "unintentionally targeted". The state of the art is demonstrated by studies on kinase inhibitors [27].

Sellers and co-workers have conducted a programme of *in vitro* ATPase assays to determine which myosins are inhibited by (\pm)-blebbistatin [28]. The ability of (\pm)-blebbistatin to inhibit proteins from the myosin classes I, V, X and XV was determined. None of these 'unconventional' myosins were inhibited to any significant degree, even at (\pm)-blebbistatin concentrations of 100 μ M [28]. Although only 5 myosin classes were assayed, the currently available data suggest that (\pm)-blebbistatin and hence (*S*)-(-)-blebbistatin (**2**) appears to be a *specific* inhibitor of myosin class II.

The activity of (\pm)-blebbistatin against different myosin II subclasses has also been investigated [28]. Rabbit *skeletal* muscle myosin, porcine β -*cardiac* muscle myosin, human NMIIA and chicken NMIIB are all inhibited by (\pm)-blebbistatin to a similar extent (IC_{50} values of 0.5, 1.2, 5.1 and 1.8 μ M respectively, Fig. 7), demonstrating limited selectivity within the myosin II class and between isoforms within the same subclass (NMII). However, turkey smooth muscle myosin (closely related to NMII) is inhibited by (\pm)-blebbistatin with an IC_{50} value of 79.6 μ M, significantly higher than that for NMIIA and NMIIB [28]. This demonstrates that (\pm)-blebbistatin does possess a degree of selective inhibition even within subclasses. However, there is considerable room for improvement providing an exciting (and challenging) opportunity for synthetic chemists who can identify subclass or isoform selective analogues.

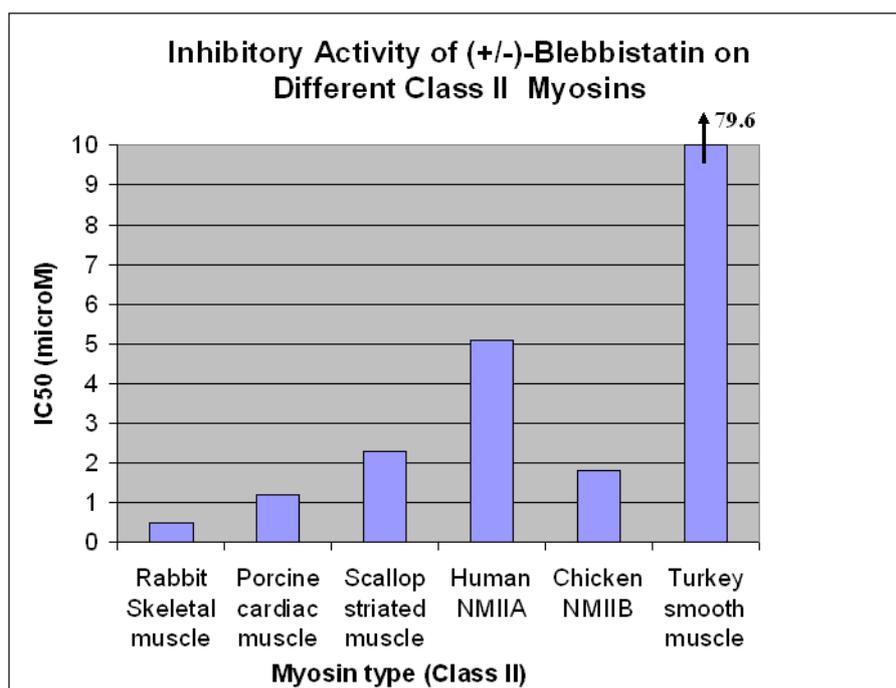


Figure 7. Assessment of (±)-blebbistatin selectivity within the myosin II class [28].

In a recent study, Ivanov and co-workers [29] were only able to determine that NMIIA was involved in the disassembly of the apical junction complex (AJC) (see following section) by determining the expression levels of the three NMII isoforms using specific antibodies. This extended set of experiments would not have been necessary if isoform selective inhibitors were available. Additionally, if the cell type used in these studies expressed two or more isoforms of NMII at similar levels no conclusion could have been drawn as to which isoform was responsible for the observed phenotype.

In addition to the myosin II class (see previous section), the unconventional myosin classes also represent important drug targets. For example class XIV consists of myosins from the *Apicomplexan* parasites *Toxoplasma gondii* and *Plasmodium falciparum* [24], both of which are important human pathogens. TgMyoA, a member of this class, has been shown to play an essential role in parasitic motility *in vitro* and in cell invasion *in vitro* and *in vivo* [30]. It can therefore be considered as a validated drug target and selective myosin XIV inhibitors as potentially novel therapeutics. In addition, specific small molecule inhibitors of host cell invasion by *T. gondii* are of use to biologists, due to the fact that the obligate intracellular nature of these parasites renders TgMyoA knockout lethal.

A recently reported HTS [31] identified a number of small molecules that inhibit host cell invasion by *T. gondii*, although it remains unclear whether any of these small molecules target TgMyoA.

(S)-(-)-BLEBBISTATIN (2) AS A MOLECULAR TOOL

To meet the needs of the cellular biology community (\pm)-, (S)-(-)-(2) and (R)-(+)-blebbistatin (8) are commercially available. Their availability has enabled researchers to investigate the role of subclasses of myosin II in cellular processes other than cytokinesis (for which there has been 3 additional reports [32-34]). Ponti *et al.* used blebbistatin (enantiomeric purity not stated) in a series of live-imaging and small molecule-based experiments [35]. These studies demonstrated that blebbistatin reduced F-actin flow in the lamella actin network, but not in the distinct lamellipodium actin network. This result is consistent with actin-dependent lamella expansion and actomyosin contraction being essential for plasma membrane protrusion and hence cellular locomotion, whilst lamellipodium protrusion is not. Experiments conducted by Grinnell and co-workers [36] using blebbistatin (enantiomeric purity not stated) suggest that platelet-derived growth factor stimulates floating fibroblast-collagen matrix contraction in a myosin-II dependent mechanism. The matrix, which serves as a model for connective tissue, was also able to contract when stimulated with lysophosphatidic acid in the presence of blebbistatin, suggesting that a different motor protein is involved in this case. Blebbistatin has also been used to study a number of other putative myosin II-dependent processes in tissue. These studies have identified a role for myosin II in the formation of apical F-actin rings and disassembly of the epithelial AJC (NMIIA, using (S)-(-)-blebbistatin (2)) [29], the severing-induced retraction of axons (using blebbistatin of unstated enantiomeric purity) [20] and the cellular migration of pancreatic adenocarcinoma (using blebbistatin of unstated enantiomeric purity) [21].

A significant advantage of (S)-(-)-blebbistatin (2) as a molecular tool is that it inhibits myosin II directly. Previous to the discovery of 2, small molecule inhibitors of the kinases that activate myosin II by phosphorylation of its regulatory light chain (MLC) had to be employed. Incomplete inhibition of the kinase activity and constitutive levels of MLC phosphorylation are significant problems with this kinase inhibitor approach [36].

LIMITATIONS OF (S)-(-)-BLEBBISTATIN (**2**) AS A MOLECULAR TOOL

A common approach to studying protein distribution (and by implication function) within a cell is to use fluorescence microscopy techniques on live cells. These experiments often rely on green fluorescent protein (GFP) fusions of the protein under investigation [37].

To visualize the fusion proteins the cells are irradiated with light of a wavelength of 420-490 nm (488 nm in confocal microscopy applications). The light emitted from the protein is collected using pass filters with a typical wavelength range of between 520 and 570 nm. However, (S)-(-)-blebbistatin (**2**) is itself fluorescent [38] limiting its use in experiments of this type. This observation was rationalized by fluorescence emission spectroscopy, which demonstrated that excitation at 440 nm resulted in significant emission by **2** in the GFP emission wavelength range [16]. To facilitate experiments that combine the need for myosin II inhibition with fluorescence imaging of live cells, it is necessary either to use an alternative fluorescent protein (e.g. red fluorescent protein) or to identify an analogue of (S)-(-)-blebbistatin (**2**) with optimized fluorescence emission properties. This serves as another example of the impact that the synthetic chemist can have in chemical genetics research. It was proposed that the addition of a nitro group to the chromophore of **2** would modify its fluorescence properties, although the addition of this functional group must be achieved without loss of biological activity. Synthesis and biological testing of analogues of (S)-(-)-blebbistatin (**2**) had previously demonstrated that substitution at C-7 could be tolerated (Lucas-Lopez *et al.*, unpublished results). Therefore, a sample of (S)-(-)-7-nitro-blebbistatin (**12**) was prepared utilizing our synthetic methodology [16]. Analysis of the fluorescence properties of **12** showed the expected reduction in fluorescence emission in the GFP wavelength range compared with **2**. Biochemical assays using **12** showed that it inhibited nonmuscle myosin IIA ATPase activity with an IC₅₀ of 28 μM.

Recent microscopy-based studies have identified a further limitation of (±)-blebbistatin [39, 40]. It was shown that prolonged exposure to filtered light (450-490 nm) results in degradation of (±)-blebbistatin to an unidentified non-inhibitory product via cytotoxic intermediates. This degradation was further investigated [16] and found to result from exposure to light in a narrow range around 436 nm.

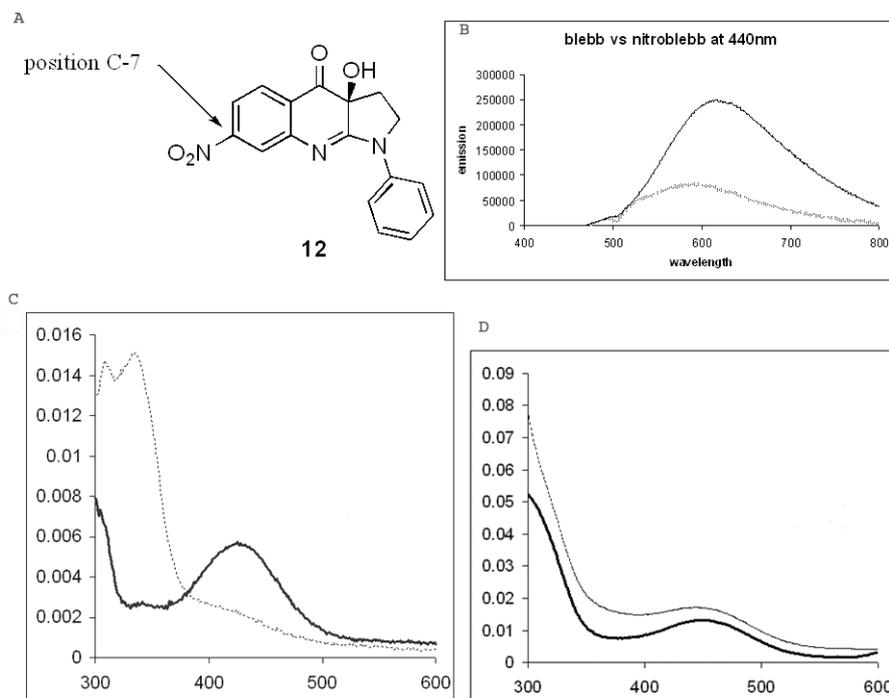


Figure 8. An analogue of (S)-(-)-blebbistatin (**2**) with optimized fluorescence properties.

We have recently shown that (S)-(-)-7-nitro-blebbistatin (**12**) is stable to prolonged irradiation in this wavelength range. This observation coupled with its reduced fluorescence and retained biological activity suggests that **12** could be a viable alternative to (S)-(-)-blebbistatin (**2**) for fluorescence imaging experiments with live cells.

FUTURE STUDIES

Biochemical experiments have shown that (\pm)-blebbistatin inhibits NMIIA ATPase activity by inhibiting the release of inorganic phosphate from the myosin/ADP/Pi complex [17,41]. This is confirmed and extended in the recently reported structure of (S)-(-)-blebbistatin (**2**) bound to the motor domain of *Dictyostelium discoideum* myosin II (Fig. 9) [42]. **2** Binds in a hydrophobic pocket at the apex of a large cleft present in the motor domain close to the γ -phosphate-binding pocket.

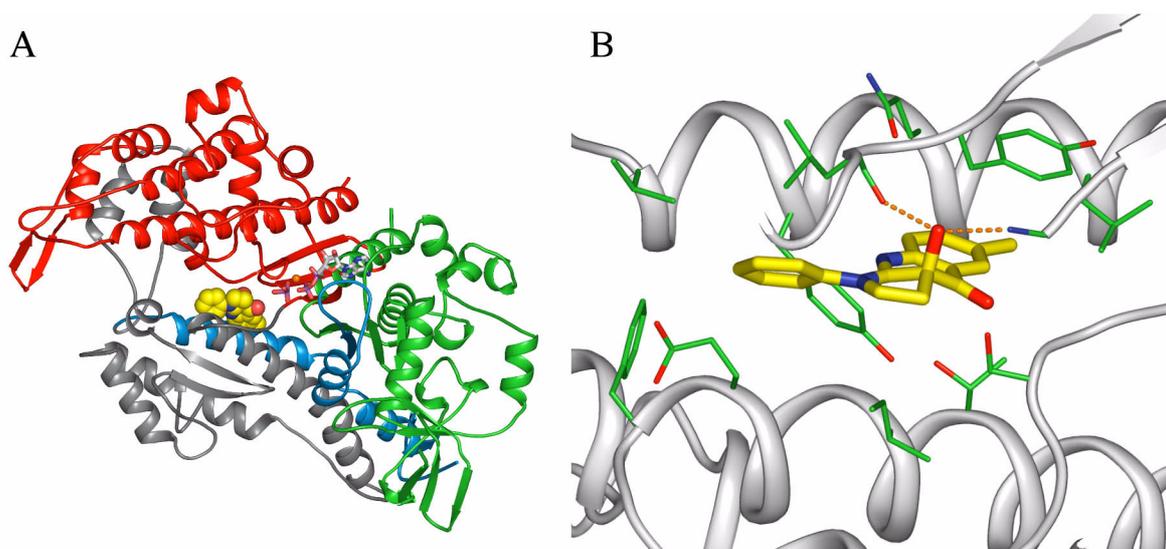


Figure 9. **A)** The structure of (*S*)-(-)-blebbistatin (**2**) bound to the MgADP-vanadate complex of *D. discoideum* myosin II. **B)** The blebbistatin-myosin binding site. Selected amino acids that interact with (*S*)-(-)-blebbistatin (**2**) are shown in green as their stick representations. For further details see reference [42].

This study opens the way for the rational design of more potent and specific inhibitors of the myosin II subclasses. Through the use of modelling studies, it should also be possible to enhance the discovery of inhibitors of other myosin classes based on the (*S*)-(-)-blebbistatin (**2**) core structure or other structures. An alternative approach to the discovery of more potent or more selective myosin II inhibitors would be to perform HTS using libraries of (*S*)-(-)-blebbistatin (**2**) analogues. Whilst the preparation of targeted libraries usually requires a significant amount of chemistry development, the advances in high-throughput synthesis and purification technology help. Our approach to this synthetic challenge involves the development of a solid supported synthesis of (*S*)-(-)-blebbistatin (**2**) analogues using a polymer supported oxidizing reagent (Blum and Westwood, unpublished results) (Fig. 10A) and the use of parallel synthesis/purification technology to prepare small focused collections (Fig. 10B-D) (Westwood, Blum and Lucas-Lopez, unpublished results). These focused libraries concentrate on incorporating substituents in positions that are known not to disrupt NMII ATPase inhibitory activity [6], (Lucas-Lopez *et al.*, unpublished results).

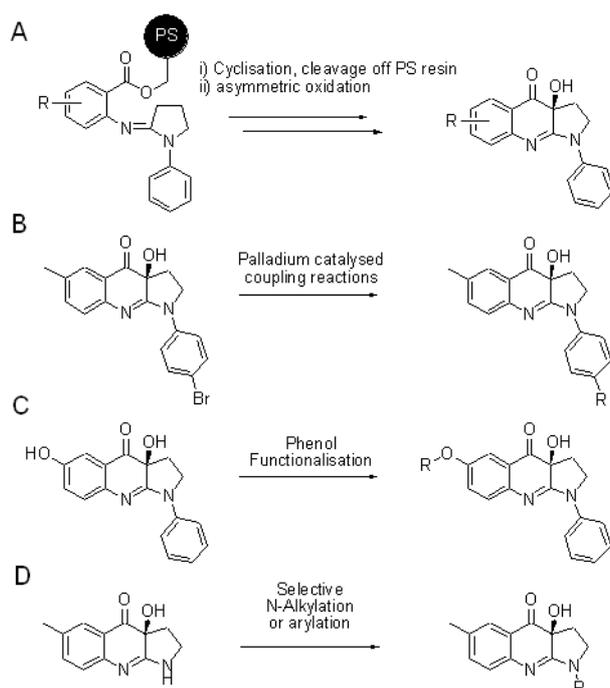


Figure 10. Current strategies for preparing targeted libraries. PS = polystyrene resin.

CONCLUSION

This article has reviewed the discovery, characterization, optimization and use of the novel small molecule tool, (S)-(-)-blebbistatin (2). It provides an overview of the challenges inherent in using chemical genetics to dissect biological mechanisms. This approach is multidisciplinary requiring expertise in cell biology, biochemistry, structural biology as well as computational and synthetic chemistry. Chemical genetics continues to have a significant impact on post-genomic science.

NOTE ADDED IN PROOF

During the editing of this manuscript several further articles describing the use of blebbistatin have appeared [43-52].

ACKNOWLEDGEMENTS

The majority of the research discussed in this article was carried out at the Institute of Chemistry and Cell Biology, Harvard Medical School, U.S.A. (A. F. Straight, T. J. Mitchison, N. J. Westwood); the Laboratory of Molecular Cardiology, National Heart, Lung and Blood Institute, National Institutes of Health, U.S.A. (J. R. Sellers, J. Toth) and the School of Chemistry, University of St Andrews (N. J. Westwood, C. Lucas-Lopez, S. Patterson, T. Blum, A. Slawin). We would like to thank Professor Ivan Rayment, Department of Biochemistry, University of Wisconsin for helpful discussions and the original versions of the images included in Fig. 9. We would also like to acknowledge the Beilstein Institut for the opportunity to take part in their 2004 symposium entitled "The Chemical Theatre of Biological Systems".

REFERENCES

- [1] Begley, T.J., Samson, L.D. (2004) Network responses to DNA damaging agents. *DNA Repair* **3**:1123-1132.
 - [2] Heath, J.R., Phelps, M.E., Hood, L. (2003) NanoSystems biology. *Mol. Imaging Biol.* **5**(5):312-325.
 - [3] Politz, J.C. (1999) Use of caged fluorochromes to track macromolecular movement in living cells. *Trends Cell Biol.* **9**(7):284-287.
 - [4] Mayer, T.U., Kapoor, T.M., Haggarty, S.J., King, R.W., Schreiber, S.L., Mitchison, T.J. (1999) Small-molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. *Science* **286**(5441):913-914.
 - [5] Westwood, N.J. (2004) Chemical Genetics: how does it function? *Phil. Trans. R. Soc. Lond. A* **362**: 761-2774 and references therein.
 - [6] Straight, A.F., Cheung, A., Limouze, J., Chen, I., Westwood, N.J., Sellers, J.R., Mitchison, T.J. (2003) Dissecting temporal and spatial control of cytokinesis with a myosin II inhibitor. *Science* **299**:1743-1747.
 - [7] Peterson, J.R., Mitchison, T.J. (2002) Small molecules, big impact: a history of chemical inhibitors of the cytoskeleton. *Chem. Biol.* **9**:1275-1285.
 - [8] Coue, M., Brenner, S.L., Spector, I, Korn E.D. (1987) Inhibition of actin polymerisation by latrunculin A. *FEBS Lett.* **213**:316-318.
 - [9] Mabuchi, I., Okuno, M. (1977) The effect of myosin antibodies on starfish blastomeres. *J. Cell Biol.* **74**:251-263.
 - [10] Ostap, E.M. (2002) 2,3-Butanedione monoxime (BDM) as a myosin inhibitor. *J. Muscle Res. Cell Motil.* **23**:305-308.
-

-
- [11] Higuchi, H., Takemori, S. (1989) Butanedione monoxime suppresses contraction and ATPase activity of rabbit skeletal muscle. *J. Biochem.* **105**:638-643.
- [12] Cheung, A., Dantzig, J.A., Hollingworth, S., Baylot, S.M., Goldman, Y.E., Mitchison, T.J., Straight, A.F. (2002) A small-molecule inhibitor of skeletal muscle myosin II. *Nature Cell Biol.* **4**:83-88.
- [13] Straight, A.F., Field, C.M., Mitchison, T.J. (2005) Anillin binds nonmuscle myosin II and regulates the contractile ring. *Mol. Biol. Cell* **16**(1):193-201 and references therein.
- [14] Henri, J.F. George, B.A., John, K.N., Austen, M.A. (2001) WPO patent #WO0121796.
- [15] Talaga, P. (2004) Compound decomposition: a new drug discovery tool? *Drug Discov. Today* **9**(2):51-53.
- [16] Lucas-Lopez, C., Patterson, S., Blum, T., Straight, A.F., Toth, J., Slawin, A.M.Z., Mitchison, T.J., Sellers, J.R., Westwood, N.J. (2005) Absolute stereochemical assignment of the small molecule tool, (-)-blebbistatin. *Eur. J. Org. Chem.* in press.
- [17] Kovacs, M., Toth, J., Hetenyi, C., Malnasi-Csizmadia, A., Sellers, J.R. (2004) Mechanism of blebbistatin inhibition of myosin II. *J. Biol. Chem.* **279**:35557-35563.
- [18] Davis, F.A., Chen, B.-C. (1992) Asymmetric hydroxylation of enolates with *N*-sulfonyloxaziridines. *Chem. Rev.* **92**:919-934.
- [19] Davis, F.A., Weismiller, M.C., Murphy, C.K., Reddy, T., Chen, B.-C. (1992) Chemistry of oxaziridines. 18. Synthesis and enantioselective oxidations of the [(8,8-dihalocamphoryl)sulfonyl]oxaziridines. *J. Org. Chem.* **57**:7274-7285.
- [20] Gallo, G. (2004) Myosin II activity is required for severing-induced axon retraction in vitro. *Exp. Neurol.* **189**:112-121.
- [21] Duxbury, M.S., Ashley, S.W., Whang, E.E. (2004) Inhibition of pancreatic adenocarcinoma cellular invasiveness by blebbistatin: a novel myosin II inhibitor. *Biochem. Biophys. Res. Commun.* **313**:992-997.
- [22] Graves, P.R., Kwiek, J.J., Fadden, P., Ray, R., Hardeman, K., Coley, A.M., Foley, M., Haystead, T.A. (2002) Discovery of novel targets of quinolone drugs in the human purine binding proteome. *Mol. Pharmacol.* **62**(6):1364-1372.
- [23] Sellers, J.R. (2000) Myosins: a diverse superfamily. *Biochim. Biophys. Acta* **1496**:3-22.
- [24] Berg, J.S., Powell, B.C., Cheney, R.E. (2001) A millennial myosin census. *Mol. Biol. Cell* **12**:780-794.
- [25] Hodge, T., Cope, M.J.T.V. (2000) A myosin family tree. *J. Cell Sci.* **113**:3353-3354.
- [26] Golomb, E., Ma, X., Jana, S.S., Preston, Y.A., Kawamoto, S., Shoham, N.G., Goldin, E., Conti, M.A., Sellers, J.R., Adelstein R.S. (2004) Identification and characterisation of nonmuscle myosin II-C, a new member of the myosin II family. *J. Biol. Chem.* **279**:2800-2808.
- [27] Davies S.P., Reddy, H., Caivano, M., Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**(1):95-105.
-

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-
- [28] Limouze, J., Straight, A.F., Mitchison, T., Sellers, J.R. (2004) Specificity of Blebbistatin, an inhibitor of myosin II. *J. Muscle Res. Cell Motil.* **25**:337-341.
- [29] Ivanov, A.I., McCall, I.C., Parkos, C.A., Nusrat, A (2004) Role for actin filament turnover and a myosin II motor in cytoskeletal-driven disassembly of the epithelial apical junction complex. *Mol. Biol. Cell.* **15**:2639-2651.
- [30] Meissner, M., Schlüter, D., Soldati, D. (2002) Role of *Toxoplasma gondii* myosin A in powering parasite gliding and host cell invasion. *Science* **298**:837-840 and references therein.
- [31] Carey, K.L., Westwood, N.J., Mitchison, T.J., Ward, G.E. (2004) A small-molecule approach to study invasive mechanisms of *Toxoplasma gondii*. *Proc. Natl. Acad. Sci. USA* **101**:7433-7438.
- [32] Rosenblatt, J., Cramer, L.P., Baum, B., McGee, K.M. (2004) Myosin II-dependent cortical movement is required for centrosome separation and positioning during mitotic spindle assembly. *Cell* **117**:361-372.
- [33] Uetake, Y., Sluder, G. (2004) Cell cycle progression after cleavage failure: mammalian somatic cells do not possess a "tetraploidy checkpoint." *J. Cell Biol.* **165**:609-615.
- [34] Pielak, R.M., Gaysinskaya, V.A., Cohen, W.D. (2004) Formation and function of the polar body contractile ring in *Spinsula*. *Devel. Biol.* **269**:421-432.
- [35] Ponti, A., Machacek, M., Gupton, S.L., Waterman-Storer, C.M., Danuser, G. (2004) Two distinct actin networks drive the protrusion of migrating cells. *Science* **305**:1782-1786.
- [36] Abe, M., Ho, C-H., Kamm, K.E. Grinnell, F. (2004) Different molecular motors mediate platelet-derived growth factor and lysophosphatidic acid-stimulated floating collagen matrix contraction. *J. Biol. Chem.* **278**:47707-47712.
- [37] Ehrhardt, D. (2003) GFP technology for live cell imaging. *Curr. Opin. Plant. Biol.* **6**:622-628.
- [38] Sakamoto, T., Limouze, J., Combs, C.A., Straight, A.F., Sellers, J.R. (2005) Blebbistatin, a myosin II inhibitor, is photoinactivated by blue light. *Biochemistry* **44**(2):584-588.
- [39] Kolega, J. (2004) Phototoxicity and photoinactivation of blebbistatin in UV and visible light. *Biochem. Biophys. Res. Commun.* **320**:1020-1025.
- [40] Sakamoto, T., Limouze, J., Combs, C.A., Straight, A.F., Sellers, J.R. (2005) Blebbistatin, a myosin II inhibitor, is photoinactivated by blue light. *Biochemistry* **44**:584-588.
- [41] Ramamurthy, B., Yengo, C.M., Straight, A.F., Mitchison, T.J., Sweeney, H.L. (2004) Kinetic mechanism of blebbistatin inhibition of nonmuscle myosin IIB. *Biochemistry* **43**:14832-14839.
- [42] Allingham, J.S., Smith, R., Rayment, I. (2005) The structural basis of blebbistatin inhibition and specificity for myosin II. *Nature Struct. Mol. Biol.* Advance online publication (doi:10.1038/nsmb908).
-

-
- [43] Yoshizaki, H., Ohba, Y., Parrini, M.C., Dulyaninova, N.G., Bresnick, A.R., Mochizuki, N., Matsuda, M. (2004) Cell type-specific regulation of RhoA activity during cytokinesis. *J. Biol. Chem.* **279**:44756-44762.
- [44] Griffin, M.A., Sen, S., Sweeney, H.L., Discher, D.E. (2004) Adhesion-contractile balance in myocyte differentiation. *J. Cell Sci.* **117**:5855-5863.
- [45] Bastian, P., Lang, K., Niggemann, B., Zaenker, K.S., Entschladen F. (2005) Myosin regulation in the migration of tumor cells and leukocytes within a three-dimensional collagen matrix. *Cell. Mol. Life Sci.* **62**:65-76.
- [46] Shu, S., Liu, X., Korn, E.D. (2005) Blebbistatin and blebbistatin-inactivated myosin II inhibit myosin II-dependent processes in *Dictyostelium*. *Proc. Nat. Acad. Sci. USA* **102**:1472-1477.
- [47] Ekman, M., Fagher, K., Wede, M., Stakeberg, K., Arner A. (2005) Decreased phosphatase activity, increased Ca^{2+} sensitivity, and myosin light chain phosphorylation in urinary bladder smooth muscle of newborn mice. *J. Gen. Physiol.* **125**:187-196.
- [48] Wong, C., Stearns, T. (2005) Mammalian cells lack checkpoints for tetraploidy, aberrant centrosome number, and cytokinesis failure *BMC. Cell. Biol.* Advance online publication (doi:10.1186/1471-2121-6-6).
- [49] Wilkinson, S., Paterson, H.F., Marshall, C.J. (2005) Cdc42-MRCK and Rho-ROCK signalling cooperate in myosin phosphorylation and cell invasion. *Nature Cell. Biol.* **7**:255-261.
- [50] Di Ciano-Oliveira, C., Lodyga, M., Fan, L., Szaszi, K., Hosoya, H., Rotstein, O.D., Kapus, A. (2005) Is myosin light chain phosphorylation a regulatory signal for the osmotic activation of the $Na^+K^+-2Cl^-$ cotransporter. *Am. J. Physiol. Cell Physiol.* Advance online publication (doi:10.1152/ajpcell.00631.2004).
- [51] Xia, D., Stull, J.T., Kamm, K.E. (2005) Myosin phosphatase targeting subunit 1 affects cell migration by regulating myosin phosphorylation and actin assembly. *Exp. Cell Res.* **304**:506-517.
- [52] Ivanov, A.I., Hunt, D., Utech, M., Nusrat, A., Parkos, C.A. (2005) Differential roles for actin polymerisation and a myosin II motor in assembly of the epithelial apical junction complex. *Mol. Biol. Cell.* Advance online publication.
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