Dissecting Temporal and Spatial Control of Cytokinesis with a Myosin II Inhibitor

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Supporting Online Material

Methods

Protein purification
Non-muscle myosin II was purified from expired human platelets according to the method of Daniel and Sellers (1) followed by gel filtration through potassium iodide and Mg•ATP as described by Pollard (2) to remove residual actin. Papain cleavage to generate subfragment 1 (S1) was performed as described (3). Chicken gizzard myosin II was purified by the method of Trybus (4). Rabbit skeletal muscle myosin S1 was generously provided by Jody Dantzig, Alex Shaw and Yale Goldman. Myosin light chain kinase was purified from chicken gizzard as described (5). Actin was purified from rabbit skeletal muscle as described (6).

High throughput screening
High throughput screening for inhibitors of non-muscle myosin II ATPase was performed as previously described (7) with the following modifications. Approximately 2 µl of an ATPase reaction mixture (200 mM KCl, 50 mM Tris pH 7.9, 2 mM MgCl₂, 0.2 mM CaCl₂, 1 mM DTT, 50 µM ATP, 10 µg/ml calmodulin, 15 µg/ml MLCK, 0.2 mg/ml human platelet myosin, 0.3675 mg/ml actin, 0.01% TritonX-100) was distributed into each well of a 1536 well plate. Chemical compounds (40 nl) were transferred into wells of the plate using a steel pin array. The reaction was incubated for 1.75 hr at 37°C then 4 nl of development solution (25 mM Tris pH 7.8, 5 mM MgSO₄, 0.1 mM EDTA, 0.1 mM NaN₃, 1 mM DTT, 1 mM D-luciferin, 2.5 µg/ml luciferase) was distributed into each well
using a cartesian sprayer. Luminescence was then imaged using a telecentric lens coupled to a cooled CCD camera. All screening was performed in duplicate.

**Myosin ATPase assays**

The actin activated Mg•ATPase activity in Figure 1B was measured in a Beckman DU-640 spectrophotometer using an NADH-linked assay (8). The conditions were 2 mM MgCl₂, 10 mM MOPS, 0.1 mM EGTA, 1 mM DTT, 20 µM actin, 200 µM NADH, 1 µM phophoenolpyruvate, 40 U/ml lactate dehydrogenase, 200 U/ml pyruvate kinase, 37° for smooth muscle HMM (9), NMIIA HMM (10), NMIIB HMM (11) and Myo1b (Mike Ostap). Rabbit skeletal muscle HMM (gift of Earl Homsher, UCLA) was measured under the same conditions, but at 25°. MyoV S1 (12) and myo10 HMM (Chen et al. in preparation) were measured at 25° under the same ionic conditions, but 0.1 M KCl was present. The activities in the presence of 50 mM and 100 mM blebbistatin were normalized to the value for the same myosin measured in the absence of blebbistatin. Each value represents the mean and S.D. of three to five assays. Myosin ATPase assays in Figures 1A and S2A were performed as described (7) with the following modifications. Platelet S1 myosin was assayed at a final concentration of 52 µg/ml in assay buffer (200 mM KCl, 10 mM Tris-HCl pH 7.0, 10 mM CaCl₂, 2 mM MgCl₂, 1 mM DTT, 0.2 mM 2-amino-6-mercapto-7methyl-purine riboside (MESG), 10U/ml purine nucleoside phosphorylase (PNP)). Myosin in assay buffer was added to a 10X stock of blebbistatin in DMSO followed by the addition of ATP to 0.1 mM. Absorbance readings at 360 nm were taken every 10 seconds. Full-length human platelet myosin and chicken gizzard myosin were assayed in the presence of 15.2 µg/ml MLCK and 10 µg/ml calmodulin. Rabbit skeletal myosin S1 was assayed at a concentration of 25 µg/ml.

**Motility assay**

*In vitro* motility was performed using full-length human platelet myosin as described (7, 13). Myosin was introduced into flow chambers at 0.23 mg/ml. The final motility reaction also contained 10 µg/ml MLCK, 5.6 µg/ml calmodulin and blebbistatin.
**Blebbistatin synthesis**

Blebbistatin was synthesized by condensation of methyl 2-amino-5-methylbenzoate with 1-phenyl-2-pyrrolidinone followed by cyclization in the presence of lithium bis(trimethylsilyl) amide. The product was then oxidized with either (1S)-(+) or (1R)-(−)-(camphorsulfonyl)oxaziridine to give blebbistatin. Detailed synthesis information will be published elsewhere (Straight and Westwood, manuscript in preparation). Based upon a related synthetic strategy (14) we believe the (−)-enantiomer to be the S-enantiomer but we have not yet confirmed the absolute stereochemistry. Enantiomers of blebbistatin were purified by isocratic elution in 9:1 hexanes:isopropanol using chiral solid phase HPLC (Chiralcel-OD-H, Daicel, Japan). Blebbistatin concentrations were normalized using an extinction coefficient of 7400 at 422 nm.

**Myosin light chain kinase assay**

Myosin light chain kinase activity was assayed in MLCK buffer (50 mM Tris pH 7.0, 100 mM KCl, 10 mM MgCl₂, 0.2 mM CaCl₂, 1 mM DTT). Each reaction contained 1 µg human platelet myosin, 0.125 µg calmodulin and 38 ng MLCK. Reactions were mixed with indicated concentrations of inhibitor then ATP was added to give a final concentration of 30 µM cold ATP and 1.5 µM g-32P-ATP (10 Ci/mmol). Reactions were incubated for 10 minutes at 30°C, stopped by the addition of Laemmli sample buffer and separated by SDS-PAGE (15%).

**Microscopy**

Microscopy using cultured M2 cells, goldfish keratocytes, and HeLa cells was performed on a Nikon TE300 inverted microscope. Cells were maintained at constant temperature in a custom designed stage incubator. Images were captured using an ORCA-ER CCD camera (Hamamatsu Inc.) using Metamorph software (Universal Imaging Corp.). Fluorescence deconvolution microscopy was performed on a DeltaVision system (Applied Precision).
**Tissue culture**

M2 cells were cultured in MEM with Earle’s salts and 10 mM Hepes pH 7.55 supplemented with 8% calf serum and 2% fetal bovine serum (FBS). XTC cells were cultured in 70% Leibovitz’s L-15 supplemented with 10% FBS, goldfish keratocytes were isolated and cultured as described (15) but without trypsinization of scales. HeLa cells were cultured in DMEM supplemented with 10% FBS.

**Drug treatments**

To test the effect of (+) and (-) enantiomers of blebbistatin on HeLa cells, cells were synchronized for 7 hours in 100 µM monastrol then released into increasing concentrations of blebbistatin. Cells were fixed (fix buffer = 80 mM PIPES pH 6.8, 1 mM MgCl₂, 10 mM EGTA, 0.2% Triton X-100, 4% formaldehyde) after 3 hours and binucleate cells were quantified by hoechst staining. For synchronized cell experiments, HeLa cells were synchronized by the addition of 2.5 mM thymidine. Two 16 hour thymidine arrests were separated by a 9 hour release period. Cells were released from the second thymidine arrest for 4 hours followed by the addition of monastrol to 100 µM and incubation for an additional 8 hours. Monastrol arrested cells were collected by shakeoff, washed out of monastrol into 100 µM (±)-blebbistatin and plated onto poly-L-lysine coated coverslips. To assess progression of cells through mitosis in blebbistatin, cells were incubated for increasing time after mitotic block on coverslips with or without blebbistatin then fixed for 15’ at 37°C. Cells were stained for α-tubulin, myosin II, lamin, and DNA. For MG132 experiments, cells were incubated for 80 minutes in blebbistatin followed by media exchange into blebbistatin ± 100µM MG132 then fixation at increasing time after MG132 treatment. For other drug experiments, cells were incubated for 60 minutes on coverslips before exchanging the medium to medium containing blebbistatin and either, 33 µM nocodazole, 5 µM latrunculin-B, 1 µM staurosporine, or 50 µM Y-27632. Twenty minutes after media exchange, cells were fixed in 80 mM Pipes pH 6.8, 1 mM MgCl₂, 10 mM EGTA, 0.2% TritonX-100 and 4% formaldehyde for 15 minutes at 37°C. Cells were then stained for tubulin, non-muscle myosin II, anillin and DNA. The duration of C-phase in XTC cells was measured by time-lapse microscopy. Cells were allowed to initiate anaphase in the presence of
blebbistatin followed by drug washout at increasing times after anaphase onset. All cells treated with blebbistatin for <45′ after anaphase onset were able to complete cytokinesis after washout but all cells treated for >45′ failed to divide (n = 9). Some contraction could occur at times >45′ but division was unsuccessful.

References

Fig. S1. The structures of non-muscle myosin II inhibitors isolated by high throughput screening. Compounds 3 and 4 represent (±)-blebbistatin and another active derivative related to blebbistatin respectively.
**Fig. S2.**  
(A) Inhibition of human platelet myosin II mediated actin filament sliding (open circles) and human platelet myosin II S1 Mg\(\text{ATPase}\) activity (open squares).  
(B) Effect of blebbistatin on myosin light chain kinase activity.
Fig. S3. Effect of blebbistatin treatment of cultured cells. (A) Constitutively blebbing M2 cells before addition (untreated), during treatment (100 µM (±)-blebbistatin) and after washout (Blebbistatin washout) of (±)-blebbistatin. (B) Goldfish keratocytes before (Control) and after (Blebbistatin) addition of 100 µM (±)-blebbistatin.
Fig. S4. Mean time after anaphase initiation to chromosome decondensation and nuclear envelope reformation in XTC cells treated without (Control) or with (Bleb) (±)-blebbistatin (n = 10).
Fig. S5. Effect of aurora B kinase inhibitor (Compound 1, Table 1 Patent # WO0121596) on the microtubule spindle and the contractile ring in blebbistatin treated cells. Cells were treated as described for figure 3, kinase inhibitor was used at a final concentration of 50 µM.
Fig. S6. Schematic representing temporal and spatial communication pathways in cytokinesis.