Blebbistatin, a Myosin II Inhibitor, Is Photoinactivated by Blue Light

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ABSTRACT: Blebbistatin is a small molecule inhibitor discovered in a screen for inhibitors of nonmuscle myosin IIA. Blebbistatin inhibits the actin-activated MgATPase activity and *in vitro* motility of class II myosins. In cells, it has been shown to inhibit contraction of the cytokinetic ring. Blebbistatin has some photochemical properties that may affect its behavior in cells. In particular, we have found that exposure to light at wavelengths below 488 nm rapidly inactivates the inhibitory action of blebbistatin using the *in vitro* motility of myosin as an assay. In addition, the inhibition of cytokinetic ring contraction can be reversed by exposure of the cells to blue light. This property may be useful in locally reversing the action of blebbistatin treatment in a cell. However, caution should be exercised as free radicals may be produced upon irradiation of blebbistatin that could result in cell damage.

Phylogenetic analysis of the available myosin heavy chain sequences reveals the presence of at least 18 classes of myosin. The human genome contains 39 myosin genes from 12 of these classes (*1*). Within cells, myosins carry out diverse functions, including contraction of the cytokinetic ring, maintenance of cell shape, vesicle transport, endocytosis, capping of surface receptors, and maintenance of stereocilia and microvillar integrity (*2*). Given the multitude of functions performed by myosins even within a single cell, specific small molecule inhibitors of myosins are invaluable. The myosin inhibitor, blebbistatin (*3*), was discovered in a small molecule screen for inhibitors of nonmuscle myosin IIA. Specificity studies showed that it inhibited all class II myosins that were investigated, but did not inhibit myosins from classes I, V, X, and XV (*4*). Within the myosin II family, the IC_{50} values for the inhibition of the actin-activated MgATPase activity ranged from 0.4 to 80 mM. Blebbistatin also inhibited the ability of myosin II to move actin filaments in the *in vitro* motility assay.

During the course of studying the inhibition of myosin's *in vitro* motility, we discovered that blebbistatin is rapidly inactivated by exposure to blue light (488 nm). In this study, we investigate the wavelength and power dependences of this inactivation and show that the inactivation works in cells. In addition, we show that illumination of blebbistatin with blue light may cause production of free radicals that can damage myosin.

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EXPERIMENTAL PROCEDURES

Preparation of Proteins. Rabbit skeletal muscle actin (*5*) and myosin (*6*) were prepared as previously described. Heavy meromyosin (HMM)[1] was prepared by chymotryptic digestion (*7*).

In Vitro Motility Assay. In vitro motility assays were performed as described in a buffer consisting of 50 mM KCl, 20 mM MOPS, 0.1 mM EGTA, 5 mM $MgCl_2$, and 1 mM ATP with 50 mM DTT, 2.5 mg/mL glucose, 0.05 mg/mL glucose oxidase, and 2 μg/mL catalase added to retard photobleaching (*8*). Actin was labeled with either rhodamine phalloidin or a mixture of rhodamine phalloidin and Alexa-488 phalloidin as described previously (*8*). *In vitro* motility was measured using an Olympus IX70 microscope equipped for both epifluorescence and total internal reflection (TIRF) microscopy (*9*). For examination of the movement of rhodamine phalloidin-labeled F-actin, the sample was illuminated with 532 nm light and the fluorescence was observed in the epifluorescence mode. For examination of the movement of Alexa-488 phalloidin-labeled F-actin, TIRF microscopy was used with excitation at 488 nm using an argon laser (SpectroPhysics, model 163-C0201) (*9*). For experiments in which the sample was briefly exposed to various wavelengths of light, a Zeiss 510 confocal microscope was used. A field of rhodamine-labeled actin filaments was observed with illumination at 543 nm. A rectangle of 34 μm × 36 μm was exposed to different wavelengths of light at various powers for 100 ms, and the behavior of the rhodamine-labeled actin filaments was observed. The power density was calculated by the method of Cogswell and Larkin (*10*) assuming a lens transmission of 60%. A 40× 1.3NA plan-neofluor objective was used.

Live Cell Imaging. All cell-based experiments were performed on a DeltaVision Spectris system using an

[1] Abbreviations: HMM, heavy meromyosin; SD, standard deviation.

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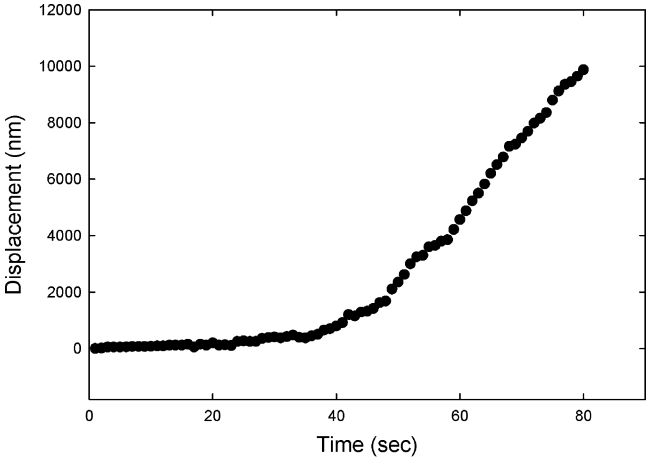

FIGURE 1: Inactivation of blebbistatin by 488 nm light. The linear displacement of the centroid position of a single rhodamine phalloidin-labeled actin filament by skeletal muscle myosin HMM is plotted as a function of time during observation with 532 nm light. The rate of actin filament sliding can be inferred from the slope.

Olympus IX70 microscope, a 40× 1.35 NA oil immersion objective, and fiber optic-coupled epifluorescence illumination and standard differential interference optics. Cells were maintained in a 37 °C water-jacketed chamber in Leibovitz L-15 with 10% fetal bovine serum during imaging. Time points were collected at 1 min intervals, and photoinactivation was accomplished by epifluorescent illumination using a 480−500 nm band-pass filter. Five (500 ms) pulses of blue light at 1 min intervals were used to photoinactivate blebbistatin. (±)-Blebbistatin was used at a concentration of 100 μM.

Reagents. A mixture of the (+) and (−) enantiomers of blebbistatin was a gift of T. Mitchison (Harvard Medical School), or the (−) enantiomer of blebbistatin was purchased from Toronto Research Chemicals.

RESULTS

Blebbistatin (100 μM) completely inhibited the movement of rhodamine phalloidin-labeled actin by skeletal muscle HMM in the *in vitro* motility assay. Actin filaments remained attached to the surface in the fully inhibited state, indicating that the inhibited molecules still retained the ability to interact weakly with actin filaments. Similar tethering behavior was seen with unphosphorylated smooth muscle myosin that did not move actin filaments, but tethered them to the surface through interactions of the weakly actin-attached myosin·ADP·P_i state with actin (*11*). This suggested that the blebbistatin-inhibited myosin interacted weakly with actin and did not interfere with the ongoing movement of the remaining uninhibited myosin. This is consistent with the observation that higher concentrations of blebbistatin are required to fully inhibit actin filament sliding than are required for inhibition of the actin-activated MgATPase activity (*3*, *4*).

While performing *in vitro* motility assays, we observed an illumination and wavelength-dependent reversal of blebbistatin inhibition. Rhodamine phalloidin-labeled actin filaments viewed by epifluorescent illumination were effectively inhibited by blebbistatin in motility assays when initially viewed, but a time-dependent reversal of the inhibition was observed (Figure 1). The reversal of inhibition required illumination, because only actin filaments in the area of the slide exposed to light resumed movement. This effect was wavelength-dependent as the motility of Alexa-488-labeled actin filaments observed with 488 nm light illumination could not be blocked with blebbistatin (Table 1). To further characterize blebbistatin's photosensitivity we co-labeled actin filaments with rhodamine phalloidin and Alexa-488 phalloidin for *in vitro* motility assays. When viewed for brief time periods with green light (λ = 532 nm) in the presence of 100 μM blebbistatin, complete inhibition of actin filament sliding by skeletal muscle HMM was observed. Remaining on the same microscopic field of view, but changing the microscope configuration to allow for 488 nm light excitation, resulted in the movement of all actin filaments within the field. The rate of blebbistatin inactivation is fast since the actin filaments were moving at control velocities as soon as the field could be examined. When the illumination was returned to 532 nm, the actin filaments slowly stopped moving over a 10−15 s period. This is too slow to be ascribed entirely to diffusion of uninhibited blebbistatin from the unilluminated solution and may reflect a slow off rate for dissociation of the inactivated blebbistatin from myosin (*12*). To determine whether blebbistatin must be bound to myosin to be photoinactivated by exposure to blue light, an *in vitro* motility flow cell lacking myosin was filled with 100 μM blebbistatin in the *in vitro* motility assay buffer, placed on the microscope stage, and exposed to 488 nm light by removing the objective to obtain a larger area of illumination. After 2 min, the solution was then added to a myosin-coated surface, and the movement of rhodamine phalloidin-labeled actin filaments was examined with green light. The actin filaments moved at near-control velocities in the samples where blebbistatin was preexposed to blue light, but not in samples that were simply incubated in the dark for the same period of time. Therefore, the photoinactivation of blebbistatin does not require the compound to be bound to myosin.

Table 1: Effect of 488 nm Light on the Blebbistatin-Induced Inhibition of Actin Filament Sliding by Rabbit Skeletal Muscle HMM

[blebbistatin] (μM)	wavelength of light used to excite actin filaments (nm)	velocity ± SD (μm/s)
0	488	4.5 ± 0.58
100	532	not detectable[a]
100	488	4.0 ± 0.51

[a] Sample was quantified within 10 s of obtaining a new field. With time the actin filaments started to move.

The photoinactivation of blebbistatin could be exploited in examining the nature of the inhibition of actin filament sliding. Using a confocal microscope, a rectangle measuring 34 μm × 36 μm was briefly exposed to 488 nm light within an observation field of 88.6 μm × 88.6 μm which was being illuminated with 543 nm light for observation of rhodamine phalloidin-labeled actin filaments. Time sequences from a portion of these images are shown in Figure 2. The yellow line represents the boundary of the 488 nm exposure with the area below the line being the portion that was exposed. Prior to the 488 nm flash, all actin filaments were immobile. Filaments entirely contained within the flashed square

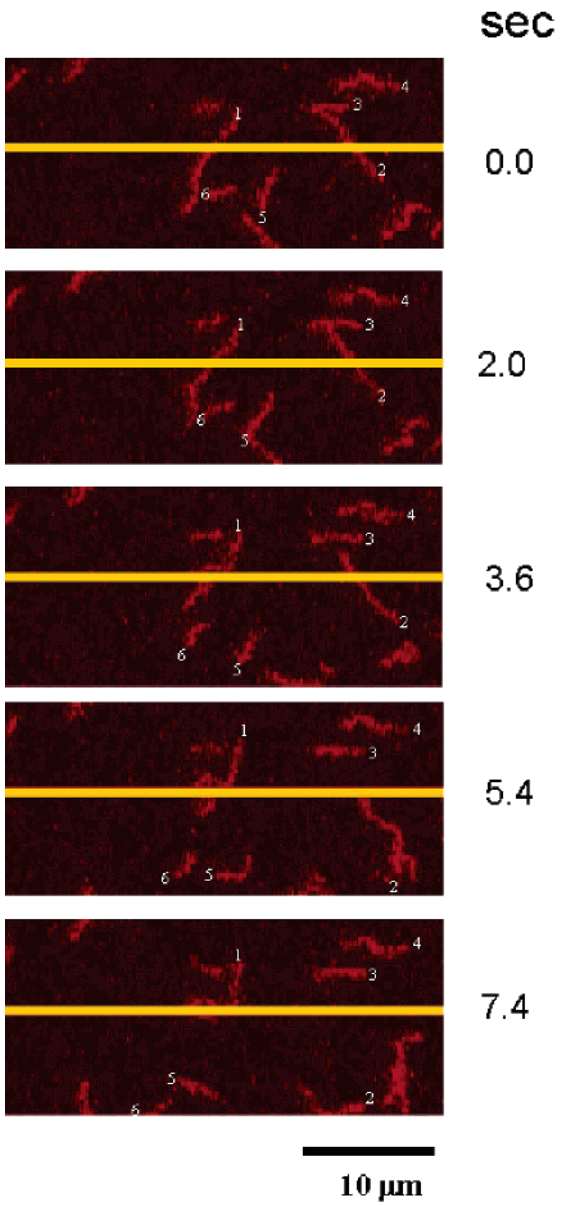

FIGURE 2: Effect of inactivation of blebbistatin on actin filament movement. Five panels of confocal microscope images of actin filaments bound to a rabbit skeletal muscle HMM-coated surface are displayed. The first panel shows blebbistatin-arrested actin filaments prior to a 100 ms flash with 230 μW of 488 nm light. In all subsequent panels, the area below the yellow line received the 488 nm light flash and the area above did not. The leading edges of six actin filaments are marked in each panel.

immediately began moving (see filaments 5 and 6), whereas filaments outside the flashed square remained immobile (filaments 3 and 4). Some filaments were initially positioned at the boundary of the region to be flashed with 488 nm light. Two distinct behaviors were observed depending on the orientation of the actin filament. Filament 1 (Figure 2) is oriented with the leading edge outside of the region to be flashed. Upon irradiation with blue light, the trailing portion of the filament moved forward, but the leading edge remained immobile. In contrast, actin filament 2 was oriented with its leading edge inside the flashed square and moved into the square with no shearing of the filament or impediment to the sliding. This is consistent with the data presented above showing that blebbistatin-inhibited myosin appears to be blocked in a weakly bound state.

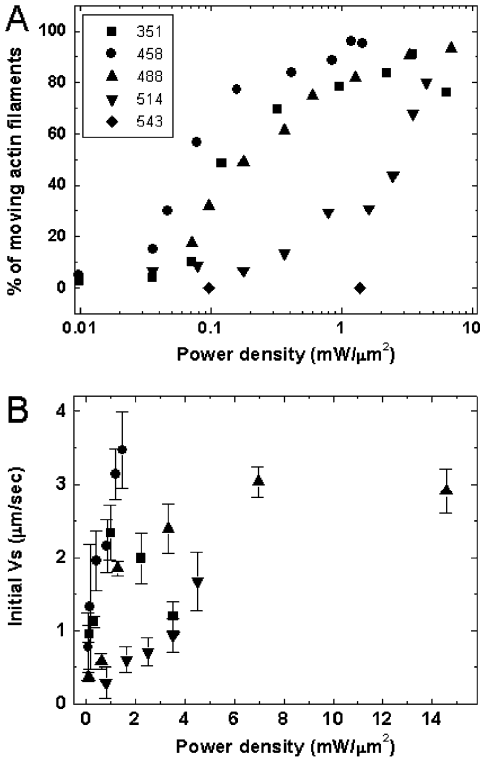

FIGURE 3: Power and wavelength dependence of blebbistatin photoinactivation. (A) A 34 μm $\times$ 36 μm rectangle of an *in vitro* motility flow chamber was exposed to the indicated wavelength of light at the indicated power for 100 ms. The percentages of rhodamine phalloidin-labeled actin filaments moving immediately upon returning to the observation wavelength of 543 nm were scored. (B) The velocity of actin filament sliding was immediately measured upon returning to the observation wavelength of 543 nm following the 100 ms flash at the indicated wavelength and power of light.

We next examined the wavelength and power dependency of this inactivation. In this experiment, several wavelengths ranging from 351 to 543 nm were used to irradiate the square described above, and the power at each wavelength was systematically varied from low power (0.01 mW/μm^2) to high power (5−14 mW/μm^2). Figure 3 shows the percentage of actin filaments that were found to be moving in the irradiated area upon returning to the observation wavelength of 543 nm. Blebbistatin was most sensitive to 458 nm light, but light at 488 and 351 nm also easily inactivated the molecule as measured by the percentage of actin filaments sliding. In contrast, a much higher power of 514 nm light was required for photoinactivation, and virtually no inactivation occurred with 543 nm light even at 1.3 mW/μm^2.

A plot of the velocity of actin filament sliding measured immediately upon return to the observation wavelength (543 nm) again demonstrated the power and wavelength dependence of the photoinactivation process (Figure 3B). The velocity of actin filament sliding upon exposure to 351 nm light showed a rise and then a decline as the power is increased. This may be due to phototoxic effects on myosin presumably due to the generation of free radicals.

To determine whether blebbistatin photoinactivation could occur in cellular systems, we irradiated blebbistatin-treated mitotic HeLa cells that contained cleavage furrows with a 500 ms pulse of 488 nm light. Untreated control cells had completed furrow contraction by 22 min after anaphase (Figure 4, Control; see movie S1 of the Supporting Informa-

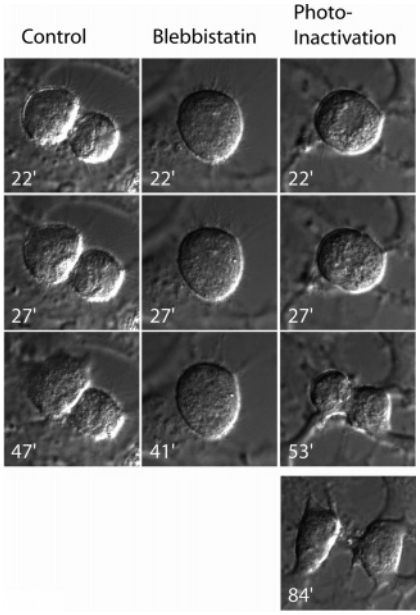

FIGURE 4: Reversal of blebbistatin inhibition of myosin II using photoinactivation in cultured HeLa cells. Untreated cells (Control), blebbistatin-treated cells (Blebbistatin), and blebbistatin-treated cells exposed to 488 nm light (Photoinactivation) were imaged during the process of cytokinesis. Minutes reflect the time after anaphase initiation. Photoinactivation was performed at time 27, and time 53 reflects the extent of contraction after photoinactivation. Blebbistatin was washed out following the 53 min observation point. Time 84 demonstrates that cells can complete contraction after blebbistatin washout.

tion), while blebbistatin-treated cells had separated chromosomes but could not contract the furrow due to myosin inhibition (Figure 4, Blebbistatin; see movie S2 of the Supporting Information). Unirradiated cells never contracted the furrow (Figure 4, Blebbistatin, 41′). We irradiated blebbistatin-treated cells with 488 nm light and observed significant contraction of the cleavage furrow after irradiation (Figure 4, Inactivation, 53′). The cleavage furrow ceased contraction, likely due to the reinhibition of myosin by active blebbistatin diffusing in from the cell culture media. To determine whether we could reverse this inhibition, we washed out the blebbistatin from the same flow cell and observed that cells completed contraction (Figure 4, inactivation, 84′; see movie S3 of the Supporting Information), suggesting that mild exposure to blue light inactivates the blebbistatin within the cell without irreversibly damaging the myosin or the cytokinetic ring apparatus. We observed significant cell toxicity upon prolonged irradiation of blebbistatin-treated cells with blue light, likely due to radical-induced photodamage (data not shown). These data suggest that blebbistatin inhibition of myosin II in cells can be reversed by illumination with blue light but that this inactivation is accompanied by phototoxicity.

DISCUSSION

Blebbistatin is a recently discovered inhibitor of nonmuscle myosin II function. It appears to be specific for class II myosins, and it potently inhibits the function of these myosins in cells. On the basis of these early results, blebbistatin appears to be a promising reagent for dissecting the specific roles of myosin II molecules in cells. However, we have found using controlled *in vitro* experiments that blebbistatin has photochemical properties when interacting with blue light that may affect its use as an inhibitor of cellular myosins.

Blebbistatin was rapidly inactivated by exposure to light below 500 nm in wavelength. The degree of inactivation was a function of both the wavelength and intensity of the light. Wavelengths below 500 nm were the most potent for inactivation. At low wavelengths and higher powers, the inactivation of blebbistatin resulted in a less than 100% recovery of the activity of myosin. This may be due to the generation of free radicals which could inactivate the myosin or affect the actin in the *in vitro* assay through chemical modification. The inactivation experiments with the *in vitro* motility assay demonstrate that blebbistatin-inhibited myosin does not offer major resistance to the sliding of actin filaments by uninhibited myosins, suggesting that myosin is trapped in a weakly bound state which is still able to tether actin filaments in the presence of blebbistatin. That blebbistatin traps myosin II in a weakly bound (with respect to actin) conformation is confirmed by transient kinetic experiments on the mechanism of blebbistatin's inhibition of myosin's MgATPase activity (*12*). Thus, blebbistatin is a "benign" inhibitor of actomyosin interactions. It does not result in strong, rigorlike binding to actin that might be expected to have very dominant effects on cells through effects on the cytoskeletal dynamics. On the other hand, to stop a cellular process driven by myosin II, it will be necessary to inhibit most of the myosin interacting with the actin filament by using blebbistatin concentrations higher than the IC_{50} values reported in this work.

The effects of blue light on blebbistatin must be taken into account when imaging cells in the presence of the drug. Clearly, one cannot study the localization of eGFP-tagged myosin II in live cells while inhibiting its action with blebbistatin. The mere act of observing the localization of the eGFP-tagged myosin with blue light would be sufficient to obliterate the inhibitory action of the drug. Such experiments will require the use of red, rather than green, fluorescent protein fusions to avoid photoinactivation of the drug.

The rapid inactivation might be a useful tool for allowing investigators to study the reversal of its effects either on whole cells or on selected areas of cells using the ability of confocal microscopes to illuminate specific patterns. However, caution must be exercised since the inactivation may be associated with phototoxicity. Since blebbistatin is very sensitive to 488 nm light, low power levels and brief exposure should be tried in any such experiment. The use of photoinactivation to reverse the effects of blebbistatin inhibition on a slow process in cells such as cytokinesis is difficult since fresh, uninactivated blebbistatin continually diffuses into the cell. While this paper was being prepared, a study that examined the phototoxicity of blebbistatin in living cells appeared (*13*). The results showed that bovine aortic endothelial cells were killed by prolonged exposure to 450−490 nm light in the presence of blebbistatin and that the effect was dependent on the concentration of blebbistatin and the time of exposure to light. Another recent study using blebbistatin on living cells found that eGFP-tagged tubulin photobleached rapidly in the presence of blebbistatin (*14*). Given the combined observations described above, blebbistatin should be used with caution, especially when cells are observed in blue light.

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SUPPORTING INFORMATION AVAILABLE

Three movies. This material is available free of charge via the Internet at http://pubs.acs.org.

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