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Research article

Rapid de-localization of actin leading edge components with BDM treatment

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Abstract

Background: 2,3-Butanedione monoxime (BDM) has been widely used as a non-muscle myosin inhibitor to investigate the role of non-muscle myosinII in the process of actin retrograde flow and other actin cytoskeletal processes. Recent reports show that BDM does not inhibit any non-muscle myosins so far tested, including nm-myosinII, prompting the question, how were these process affected in BDM studies?

Results: We have found that treatment of mammalian cells with BDM for only 1 min blocks actin incorporation at the leading edge in a permeabilized cell system. We show that inhibition of actin incorporation occurs through de-localization of leading edge proteins involved in actin polymerization – the Arp2/3 complex, WAVE, and VASP – that de-localize concomitantly with the leading edge actin network.

Conclusion: De-localization of actin leading edge components by BDM treatment is a newly described effect of this compound. It may explain many of the results previously ascribed to inhibition of non-muscle myosinII by BDM, particularly in studies of leading edge dynamics. Though this effect of BDM is intriguing, future studies probing actin dynamics at the leading edge should use more potent and specific inhibitors.

Background

The actin cytoskeleton is a dynamic system composed of actin filaments, proteins that regulate filament dynamics, and proteins that remodel and make use of the cytoskeletal network. An important aspect of actin cytoskeletal research has been the use of small molecules that affect actin dynamics and proteins that act on the cytoskeleton [1]. 2,3-butanedione monoxime (BDM) is a small molecule that inhibits the ATPase activity of muscle myosinII and reduces the force generated by this motor protein [2,3]. BDM was introduced to the cell biology community

as a millimolar inhibitor of multiple non-muscle myosins with greatest potency in inhibiting non-muscle myosinII [4]. It was subsequently used, in concert with other approaches, to study nm-myosinII function in a variety of biological processes [5–8]. BDM was recently shown not to inhibit any non-muscle myosins so far tested, leaving the question of how it affects cells unanswered [9,10].

One process inhibited by BDM is the centripetal flow of actin from the leading edge into the cell body, known as retrograde flow [11,12]. Retrograde flow is thought to be
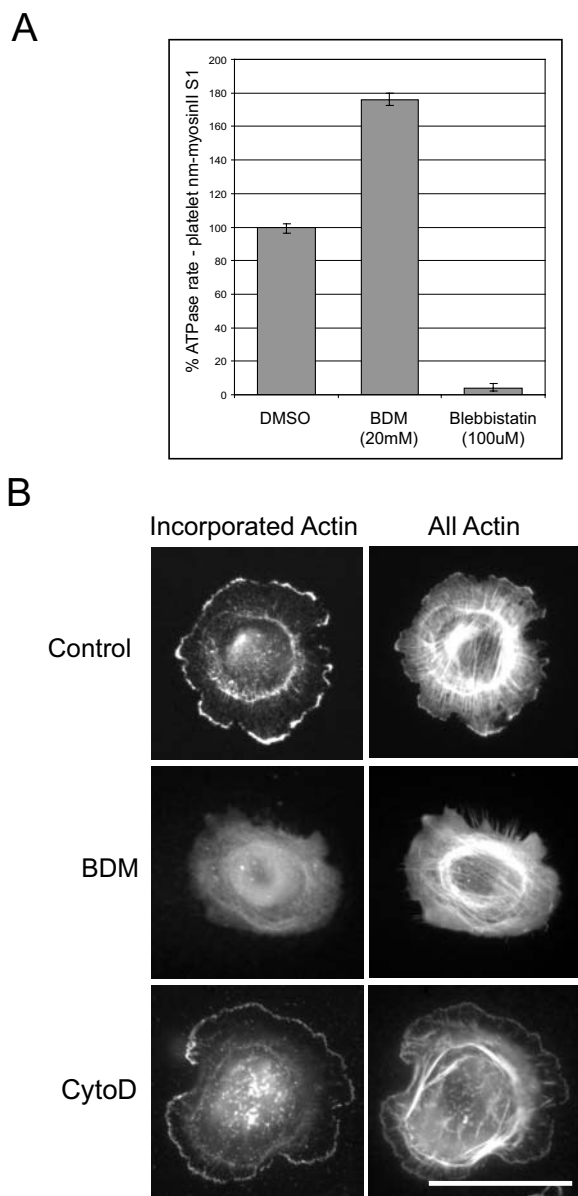
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Figure 1
BDM treatment inhibits actin incorpolration in a permeabilized cell system. (A) BDM does not inhibit platelet myosinII S1 ATPase activity. (B) Spreading Swiss3T3 cells were treated with a media control, BDM, or cytochalasin D for 1 minute prior to permeabilization and incubation with rhodamine-labeled actin. BDM treatment blocks actin incorporation at the leading edge while control and cyto D treatment have no effect. Bar, 50 um.

driven by a "push-pull" mechanism of polymerization of actin at the leading edge of the cell and contractile activity of myosin motor proteins [13,14]. Formation of new actin

filaments is predominantly mediated by the nucleation activity of the Arp2/3 complex [15]. Members of the WASP/WAVE family of proteins, among others, stimulate and regulate the Arp2/3 complex for spatial and temporal control of filament nucleation [15]. Localization of these proteins to the leading edge, though not completely understood, is probably mediated through their interactions with the actin cytoskeleton itself and membrane-associated binding partners [15,16].

Inhibition of retrograde flow by BDM has been used as evidence for the role of nm-myosins in retrograde flow [12]. Though not explicitly stated, the localization and importance of nm-myosinII in multiple cell types, and the fact that BDM was thought to have greatest potency in inhibiting nm-myosinII has led most to look at BDM's effect on retrograde flow as a result of inhibiting this protein. [11,17,18]. A role for nm-myosinII may exist, given its localization and that other perturbations that affect myosin function – myosin S1 microinjection, ML-7 treatment, and expression of dominant negative nm-myosinII constructs and have similar effects to BDM treatment [5,7,12]. However, a study in chick dorsal root ganglia neurons where nm-myosinII was inactivated using chromophore assisted laser inactivation saw no effect on retrograde flow rates [19]. We know now that BDM does not in fact inhibit nm-myosinII. So, is this effect that we see with BDM mediated by other proteins involved in retrograde flow? And what can this tell us about the mechanism of retrograde flow? How similar is this process to other actin mediated processes that use these same proteins, such as Listeria motility? BDM has a clear effect on a number of cell biological processes. Understanding how these effects are mediated can help us learn from BDM and reinterpret experiments that made use of it, while working to find new and better inhibitors.

Results
BDM inhibits actin incorporation in mammalian cells
BDM has been reported to not inhibit nm-myosinII ATPase activity [9]. We show data [Figure 1A] that supports this report. The ATPase activity of platelet nm-myosinII S1 fragment, is not inhibited by BDM while the inhibitor, blebbistatin, decreases ATPase activity by more than 90%. The stimulation of ATPase activity by BDM [Figure 1A] is similar to a ~2 fold increase seen previously (A. Straight personal communication).

Though BDM does not inhibit nm-myosinII, in permeabilized yeast (*S. cerviseae*) BDM has been shown to decrease incorporation of exogenous actin into the cytoskeleton [20]. This process of actin incorporation requires proteins involved in actin filament formation and proteins that may localize a nucleation complex [20,21]. To determine if BDM has a similar effect on actin incorporation in
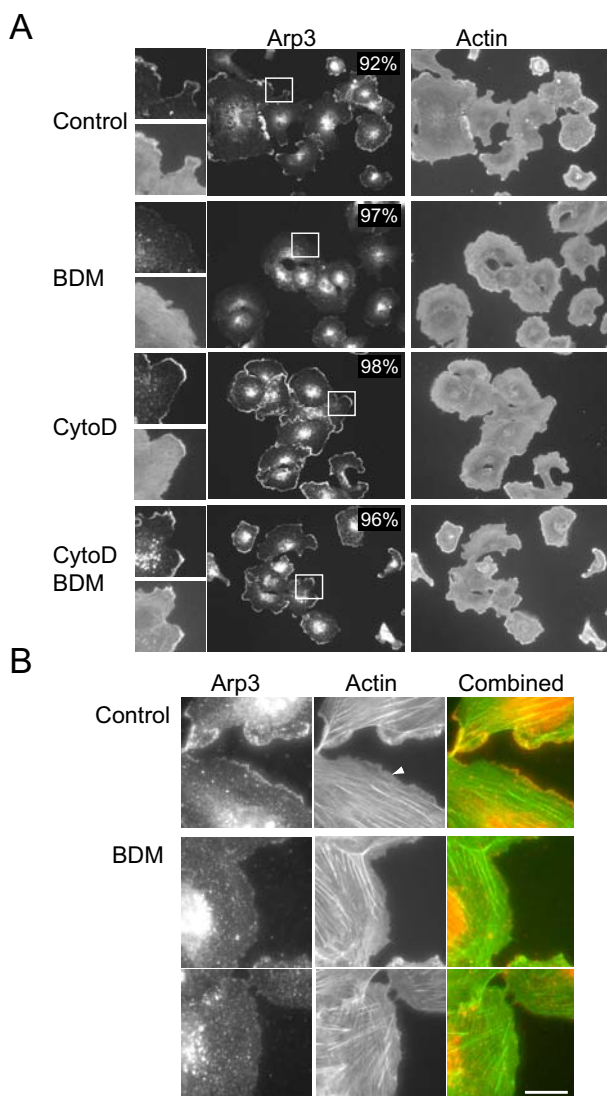
Figure 2
Arp3 is de-localized with BDM treatment. (A) BS-C-1 cells stained for Arp3 and actin after 1 minute of treatment with a media control, BDM, cytochalasin D, or both BDM and cytochalasin D. BDM treatment leads to de-localization of Arp3 and loss of the brightly stained leading edge actin network. Arp3 is not de-localized with cytochalasin D treatment or a combination of cyto D and BDM, suggesting that actin dynamics may be required for de-localization. The percentage of cells with the phenotype pictured is noted for each treatment (n =100 cells). Bar, 50 um. (B) Higher magnification of control and two BDM treated fields show actin and Arp3 staining. Actin at the leading edge of control cells (arrow) is denser, suggestive of a well-organized network, and has fewer bundled projections as compared to BDM treatment. Bar, 20 um.

mammalian cells, and if so an effect on actin nucleation, spreading Swiss3T3 cells were treated briefly with a media control or 15 mM BDM and then permeabilized with the detergent saponin in the presence of fluorescently labeled actin. The cells were then fixed and incorporation of exogenous actin was determined by fluorescence microscopy [22]. Control cells show fluorescent incorporation at the periphery, or leading edge, of the cell [Figure 1B]. Treatment of cells with 15 mM BDM for one minute blocked incorporation at the leading edge, as seen by the lack of fluorescence intensity, without significant effects on cell morphology [Figure 1B]. Cells that remained flat and were not disrupted by the permeabilization process were counted, and more than 75% (3 trials) showed decreased fluorescence similar to that seen in Figure 1B (cells that showed significant perturbation by the permeabilization process did not show greater actin incorporation). Incubation in BDM for longer than 1 minute caused more pronounced morphological change upon permeabilization, and one-minute treatment was used for subsequent experiments.

As a control, cytochalasin D, a small molecule that inhibits actin dynamics by capping the growing ends of actin filaments, was added to cells. If added prior to permeabilization but not during, exogenous actin was still able to incorporate into permeabilized cells and fluorescence incorporation was high, similar to control cells [Figure 1]. As cytochalasin D has a high affinity (50 nM) for the growing ends of actin filaments, this treatment should inhibit polymerization of existing filaments but should not block new filament formation [23]. Thus the actin incorporated most likely reflects nucleation of new filaments, as previously discussed [24]. As expected, cells treated with cytochalasin D during permeabilization showed no actin incorporation (data not shown).

To determine if BDM was affecting actin dynamics directly, we assayed its ability to inhibit *in vitro* actin assembly. No effect was seen on the polymerization of actin alone or as stimulated by VCA-activated Arp2/3 complex, using a pyrene-actin polymerization assay (data not shown). This result was supported by the observation that *Listeria monocytogenes* continue to move intracellularly, as described below.

Arp3 is no longer localized at the leading edge with BDM treatment

Since actin incorporation is not due to elongation of existing filaments and BDM does not affect pure actin dynamics in vitro, we examined the localization of Arp3, a subunit of the actin nucleation Arp2/3 complex. In spreading Swiss3T3 cells and spread BS-C-1 cells, Arp3 localized to the leading edge of the cell. However, after treatment with 20 mM BDM for one minute, localization
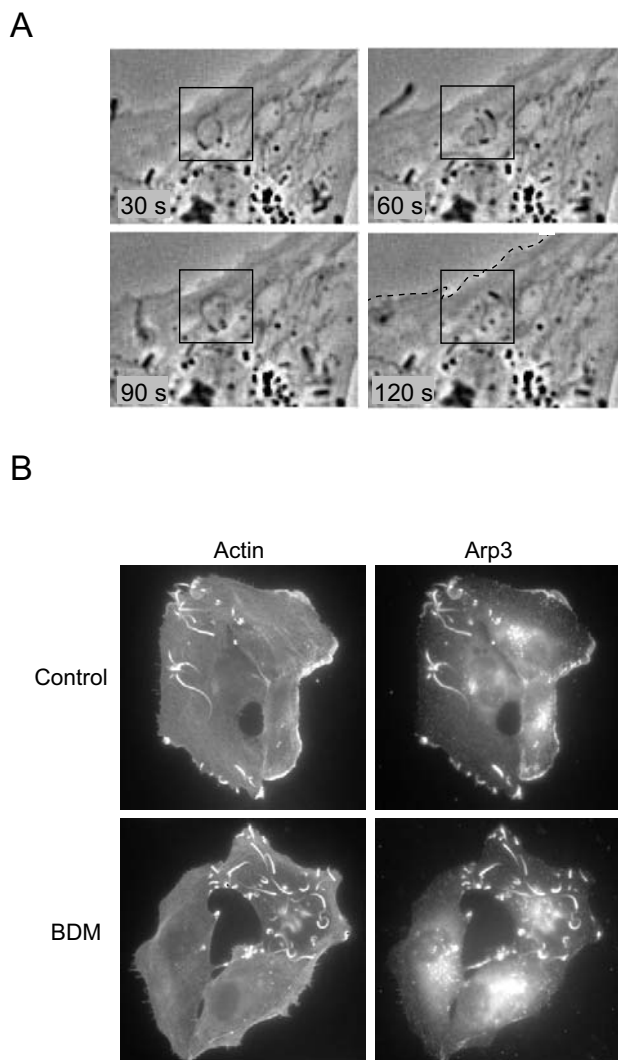
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Figure 3
Movement of *Listeria monocytogenes* and Arp3 localization in the actin comet tail is not disrupted with BDM. (A) A *Listeria* bacterium (boxed) continues to move with a phase dense actin tail after BDM addition at 0s. Inhibition of cell ruffling can be seen at 120s, the outline of the cell boundary at 0s is marked by a dotted line. (B) Localization of Arp3 in *Listeria* comet tails shows no discernible difference between control and BDM treatment. Note the lack of localization at the leading edge of the BDM treated cells.

brightly staining actin network at the leading edge [Figure 2A,2B]. Titration of BDM down to 10 mM showed the same loss of localization; however, 2 minutes of treatment was required to see equivalent loss (data not shown). To control for possible osmotic effects of BDM treatment, we treated cells with 20 mM urea, and as with a media control, more than 90% of the cells showed localization of Arp3 at the leading edge.

To determine if inhibition of actin dynamics alone was sufficient for the loss of Arp3 localization, we treated cells with cytochalasin D and examined the localization of Arp3. Within one minute at 1 uM cytochalasin D, cell ruffling ceased, yet Arp3 remained at the leading edge [Figure 2A]. In fact, Arp3 staining at the leading edge was consistently higher. Combining cytochalasin D and BDM, we saw localization equivalent to or greater than no drug treatment [Figure 2A]. Thus, actin dynamics may be required for the loss of Arp3 at the leading edge upon BDM treatment.

As mentioned, *Listeria* infected cells were used to confirm that BDM does not directly affect actin dynamics. *Listeria* exploit cellular – actin polymerization and organization – proteins to move intracellularly. We found that 20 mM BDM does not affect movement of bacteria inside treated cells [Figure 3A] consistent with a previous report [4]. Interestingly, localization of Arp3 in *Listeria* comet tails is unaffected with BDM treatment [Figure 3B] though Arp3 localization at the leading edge of these cells is disrupted.

VASP and WAVE are no longer localized to the leading edge with BDM treatment
To ask whether the loss of leading edge localization was specific to the Arp complex, we examined the localization of VASP and WAVE1 with BDM treatment. Both VASP and WAVE function in controlling actin dynamics at the leading edge – VASP is thought to promote filament elongation, and WAVE, actin nucleation [25,26]. Both VASP and WAVE1 were de-localized from the leading edge after 1 min of BDM treatment [Figure 4]. Similar to Arp3, the localization of these proteins was not disrupted by cytochalasin D treatment, or by the combination of cytochalasin D and BDM [Figure 4].

Dynamics of the loss of Arp-3 localization
Two possible mechanisms for Arp2/3 complex de-localization were considered: Arp2/3 could move back from the leading edge with retrograde flow of actin and then be lost from the filaments, or it could simply diffuse from the leading edge. To determine the dynamics of loss of Arp2/3 complex localization, we generated a stable cell line expressing Arp3-GFP. After BDM treatment, we observed a uniform decrease in fluorescence suggestive of dissociation and diffusion of Arp3, rather than de-localization
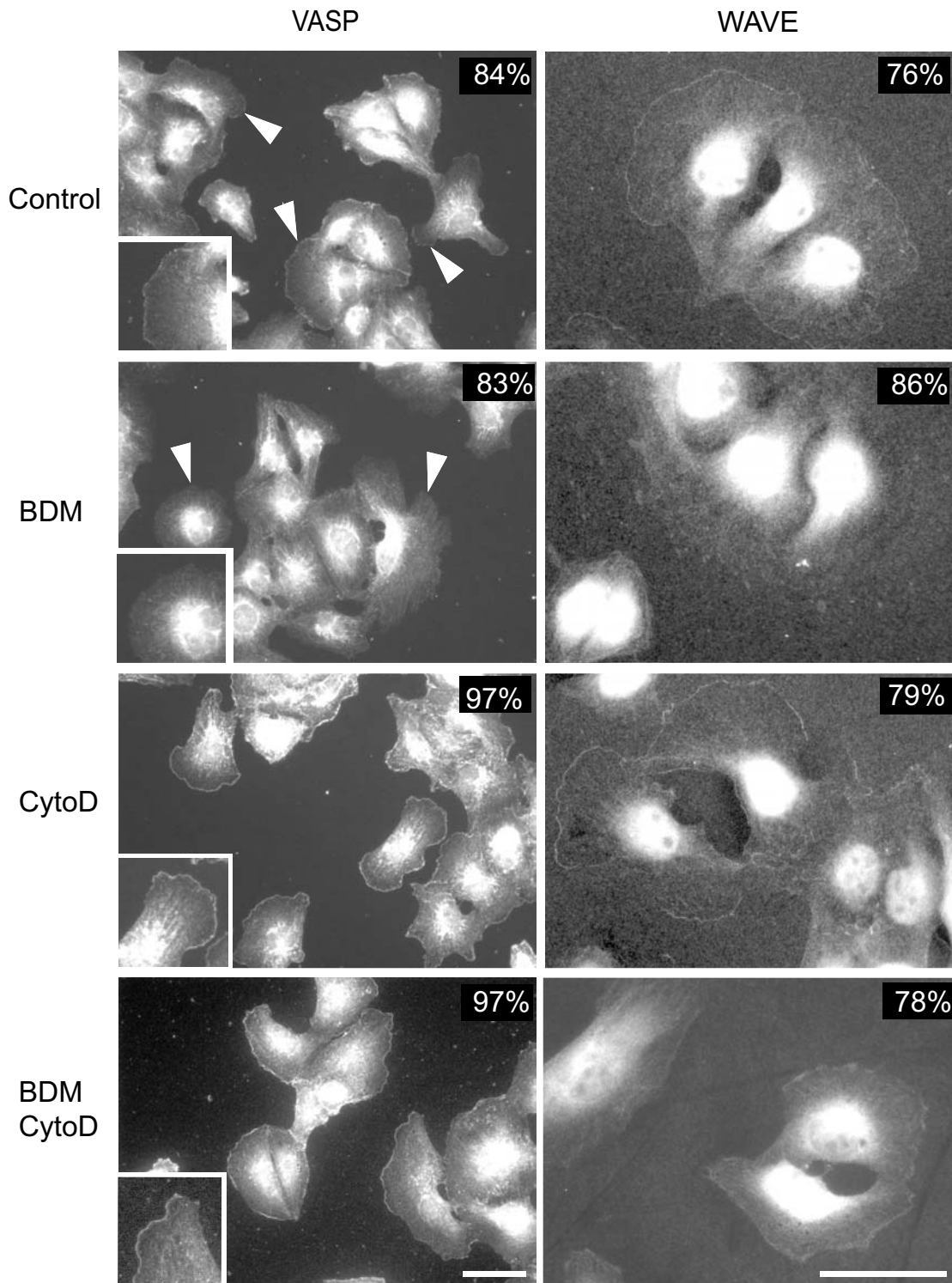
was lost in over 90% of the cells [Figure 2A]. Results for BS-C-1 and Swiss3T3 cells lines were equivalent and the results from BS-C-1 cells are shown here, as they are used in subsequent time-lapse experiments. Consistent with the inhibition of actin polymerization in the permeabilized cell assay, cells treated with BDM do not show a

Figure 4
VASP and WAVE1 are de-localized with BDM treatment. BS-C-1 cells stained for VASP or WAVE1 after treatment with a media control, BDM, cytochalasin D, or both BDM and cytochalasin D. Closed arrowheads denote the leading edge localization of VASP. The percentage of cells with the phenotype pictured is noted for each treatment (n = 100 cells). Bars, 50 um.
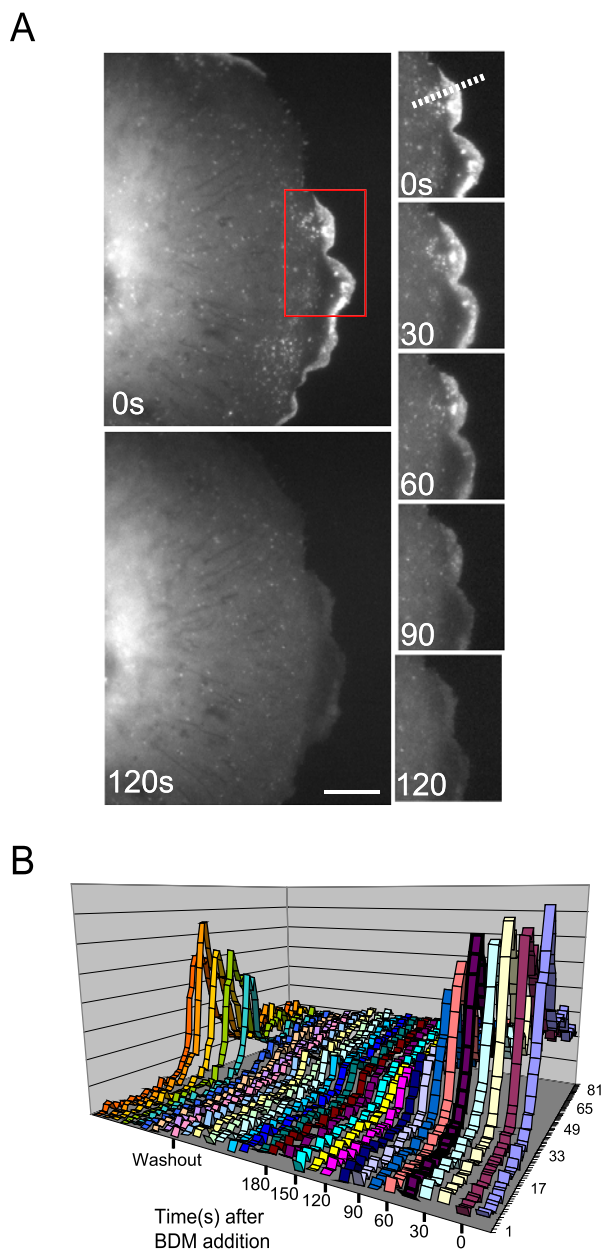
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Figure 5
Arp3 de-localization consistent with dissociation from the actin network. (A) Time-lapse sequence of a BS-C-1 cell expressing Arp3-GFP shows delocalization of Arp3 within 2 minutes of BDM addition. (B) A linescan of the area marked at 0s (dotted line) plotted over time shows gradual loss of fluorescence with only slight retraction of the leading edge. Washout brings a return of fluorescence in ~1 minute. Bar, 10 um.

driven by retrograde flow [Figure 5A]. A linescan of the leading edge shown in Figure 5A, plotted across time shows the pattern and extent of loss of fluorescence signal [Figure 5B]. Fluorescence levels decrease gradually with little or no shift in the peak of fluorescence backward. By two minutes, fluorescence levels at the leading edge are equivalent to background. After washout of BDM, Arp3-GFP localization returns in just over one minute. In all cases, the loss of Arp3-GFP at the leading edge was seen concomitantly with loss of ruffling and retraction of the leading edge or as de-localization with slight retraction. We did not see retrograde flow of the Arp3 signal in an otherwise stable leading edge.

Discussion

BDM has been used to probe actin-dependent processes on the assumption that it is a specific inhibitor of myosins. BDM has recently been found not to inhibit the *in vitro* ATPase activity of human platelet nm-myosinII, Acanthamoeba myosinIc, human myoIe, chicken myosinV, and porcine myosinVI, though it does inhibit rabbit muscle myosinII [9,10]. Thus the literature's interpretations of BDM's effects are probably incorrect. Our results suggest that the effects of BDM on actin-dependent processes such as cell motility and retrograde flow may result from effects on proteins involved in actin polymerization and the leading edge actin itself. It is not clear if these effects on leading edge components are direct or indirect. The loss of actin polymerization machinery, however, is a previously un-described effect of BDM treatment and this highlights the importance of defining the localization of proteins involved in actin dynamics when leading edge activity is disrupted.

BDM application leads to the loss of localization of leading edge components involved in actin nucleation. We observe that within two minutes, application of BDM leads to the de-localization of the leading edge components – Arp3, VASP, and WAVE – and this is concurrent with the loss of the actin network at the leading edge. Our data suggest a mechanism of de-localization of Arp3 consistent with dissociation or diffusion rather than retrograde flow. We cannot determine the causal relationship between loss of leading edge components and loss of the leading edge actin network itself. At the least, the two occur simultaneously.

Interestingly, *Listeria* bacteria continue to move in the presence of BDM, while the leading edge of the infected cell stops ruffling. Both processes have been shown to involve the Arp2/3 complex. That BDM inhibits one actin dependent process but not the other, may be an interesting difference in the location of polymerization within the cell – actin polymerization at the leading edge vs. the cell body, or a difference in the mechanism of actin

polymerization – the leading edge vs. a *Listeria* bacterium. BDM treatment thus seems to disrupt actin dynamics specific to the plasma membrane and this may provide a clue to both the mechanism of BDM inhibition and the relationship of actin dynamics at the plasma membrane.

Current understanding of the retrograde flow of actin has been influenced by the use of BDM in the sea urchin coelomocyte and *Aplysia* bag cell neuron systems. Our data are consistent with results in sea urchin coelomocytes where BDM phenocopies the effect of cytochalasin D, blocking retrograde flow at the leading edge with continued contraction of the actin network causing a clearing of actin at the leading edge [11]. In the coelomyocte system, de-localization of leading edge components by BDM could account for the inhibition of actin polymerization and clearing. In the *Aplysia* bag cell neuron, BDM application leads to extension of filopodia and the growth cone itself. Filopodial extension was interpreted as supporting the clutch model, with myosins pulling filaments toward the growth cone interior. When the myosin activity was blocked with BDM treatment, continued actin polymerization, thought to occur as elongation of existing filaments, lead to protrusion [12,27,28]. Our observation of BDM activity suggests an alternative explanation. Perhaps components required for nucleation are de-localized by BDM allowing new polymerization to be re-directed to filopodia, as filopodial extension is thought to be driven by elongation and not nucleation [27]. The extension of the growth cone itself could result from a similar diversion of actin monomer from nucleation of new filaments to extension of existing filaments. Treatment of cells with BDM often leads to filopodia-like projections as seen in Figure 1B and Figure 2B. Data from studies of *in vivo* and *in vitro* filopodia formation suggest that they develop as a reorganization of the dendritic network in which they exist [29,30]. In the presence of proteins that bundle and reorganize actin filaments into filopodial precursors, the de-localization of Arp2/3 and other leading edge components may lead to the formation of filopodia, which are then elongated as described above. Future studies will hopefully clarify the role of actin nucleation in the *Aplysia* system or alternative mechanisms that would reconcile the discrepancies between systems.

In all systems, a better understanding of how the leading edge is constructed and maintained will require specific and detailed descriptions of the activity and localization of the proteins involved. Membrane ruffling, often used as a measure of actin dynamics at the leading edge, is a very crude readout. The sensitivity of the leading edge is demonstrated by the observation that DMSO treatment at a concentration of 1% causes temporary inhibition of ruffling and concomitant Arp3 de-localization (Yarrow unpublished results). Experimental approaches that may

help address how such a dynamic and sensitive structure is established and maintained include combining inhibition of actin dynamics with the use of tools that affect Arp2/3 or myosin activity or localization, in specific ways. Though preliminary, we have seen that Arp3 is still localized in the presence of latrunculin A and jasplakinolide, and that this localization is lost with BDM treatment unlike the combination of cytochalasin D and BDM (Yarrow unpublished results). These results may in the end tell us more about our understanding of what latrunculin A and jasplakinolide do *in vivo* than the leading edge itself. Regardless, this type of experiment awaits more specific tools than BDM for follow up.

Though BDM has a relatively high effective concentration (in the millimolar range), it was widely adopted by the cell biology community because of its rapid and reversible effect on cell behaviour. BDM has not been exhaustively tested on all myosins, and a myosin target is not unreasonable given findings in Acanthomoeba and *S. cerviseae* though it does not inhibit Acanthamoeba myosinIc or human myoIe *in vitro* [10,20,21,31]. Regardless, we and others have shown that BDM is not the tool that should be used to investigate these behaviours. The small molecule approach continues to be an effective means to develop tools for understanding cell biological process when used and interpreted appropriately. One new compound that will help clarify the role of nm-myosinII is blebbistatin [32]. Other small molecules wait to be found and experiments using them will continuously refine our understanding of the leading edge and the mechanism of action of these compounds.

Conclusions
De-localization of actin leading edge components by BDM treatment is a newly described effect of this compound. It may explain results previously ascribed to inhibition of non-muscle myosinII by BDM, particularly in retrograde flow studies. Though this effect of BDM is intriguing, future studies that look at the establishment and maintenance of the actin leading edge should use more potent and specific inhibitors.

Methods
Platelet myosinII ATPase assay
The assay was performed using an EnzCheck phosphate release assay (Molecular Probes). Platelet myosinII S1 was diluted to 0.050 mg/ml in reaction buffer (0.2 M KCl, 10 mM Tris pH 7.0, 10 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM DTT, 0.2 mM 2-amino-6-mercapto-7-methyl purine riboside (MESG) in the presence of 1 U purine nucleoside phosphorylase (PNP)). DMSO, 20 mM BDM, or 100 uM blebbistatin was added to the reactions with a constant DMSO concentration of 1%. ATP (100 uM) was added to initiate the reaction and absorbance at 360 nm was read every ~6

seconds for 20 minutes. BDM showed no effect on ATP hydrolysis in the absence of myosin.

Tissue Culture
BS-C-1 cells and Swiss3T3 cells were acquired from the ATCC (CCL-26, CCL-92). Cells were maintained in Dulbecco's Modified Essential Medium with 4.5 g/L glucose, 10% fetal calf serum, and penicillin/streptomycin. Cells were maintained at 37°C with 5% CO_2.

Permeabilized cell assay
Permeabilized cell assays were performed in flow chambers as previously described [22]. Swiss3T3 cells were flowed into the chamber, the chamber inverted, and cells allowed to spread for 30–45 minutes in a humidified environment at 37°C, 5% CO_2. The assay was performed by adding 50 ul of the warmed sample solution to the chamber while wicking with Whatman paper. Cells were rinsed with PBS, treated with compounds or media for 1 min, permeabilized in the presence or absence of compound for 3 min with permeabilization buffer (0.02 g/ml of saponin, 20 mM HEPES (pH 7.5), 138 mM KCl, 4 mM $MgCl_2$, 3 mM ETGA, 1 mM DTT + protease inhibitors, 0.4 uM rhodamine actin, 1 mM ATP), and fixed in PBS + 3.7% formaldehyde for 10 min.

Listeria infection
Listeria monocytogenes (strain 10403S) were grown overnight in 3 mls of Brain Heart Infusion media at room temp without shaking. BS-C-1 cells on 25 mm poly-L-lysine (PLL) coverslips were rinsed with media containing no antibiotics and placed in a 6 well plate. *Listeria* were prepared by removing 1 ml of the overnight culture, pelleting by a 1 min Eppendorf centrifuge spin, washing the pellet twice with antibiotic-free media, and resuspending in 1 ml of media. 10 ul of Listeria were added to each well and were allowed to infect for 1.5 hrs prior to the addition of media containing 50 ug/ml Gentamicin. Cells were imaged between 4 hrs and 12 hrs later.

Compound treatment for immunofluorescence
Cells were allowed to spread on 18 mm round PLL coverslips in a 12 well plate for 30–45 minutes or 2–4 hours prior to treatment. BDM (Calbiochem 203984) stock solution was made in media at 0.5 M and Cytochalasin D (Sigma C8273) in DMSO at 20 mM. With the plate on a 37°C heat block, appropriately diluted concentrations of each compound (15 or 20 mM BDM, 1 uM cytochalasin D) were made in warmed media and added to cells after removal of existing media. After 1 minute, the cells were fixed for immunoflourescence as described below.

Immunofluorescence
Cells were fixed in a permeabilization/fixation buffer (100 mM K-Pipes pH 6.8, 10 mM EGTA, 1 mM MgCL2,

0.2% TritonX-100, 3.7% formaldehyde) for 10 minutes. For Arp3 immunofluorescence, cells were post-fixed in methanol (-20°C) for 2 minutes. Coverslips were rinsed with TBS containing 0.1% Triton X-100 (TBS-Tx) and blocked in TBS-Tx with 2% BSA (AbDil) for 45 minutes. Antibodies were diluted in AbDil and incubated for 45 minutes. Coverslips were rinsed in TBS-Tx and incubated with fluorescently labeled secondary antibodies in TBS-Tx for 45 minutes. Coverslips were rinsed and mounted in 90% glycerol with 0.5% p-phenylenediamine in 20 mM Tris-pH 8.8.

Antibodies used: rabbit Arp3 antibodies (gift from C. Egile using the same peptide as described [33]) used at 1:500 dilution, mouse actin antibodies (Santa Cruz SC-8432) used at 1:10,000 dilution, rabbit VASP antibodies (gift from F. Southwick) used at 1:100, rabbit WAVE1 antibodies (gift from S. Eden as described [26]) used at 1:100 and Alexa-dye labeled secondary antibodies, goat anti-rabbit and goat anti-mouse (Molecular Probes).

GFP-Arp3 cell line
A stable BS-C-1 cell line was made by transfection of GFP-Arp3 (gift from D. Schafer as described [34]) using Fugene6 (Roche) and selection with 500 ug/ml Geneticin. GFP expressing colonies were picked and expanded.

Live imaging of GFP-Arp3
GFP-Arp3 cells were split onto 25 mm PLL coated coverslips 4–6 hrs before imaging. Cells were transferred to Liebovitz L-15 media with 10% fetal calf serum in a heated (37°C) open-air chamber 15 minutes prior to imaging. Images were captured on a Nikon TE300 inverted microscope with appropriate neutral density filtering using an Apo 100 × /1.4 NA oil emersion phase objective and captured on a CCD camera (Hammamatsu-OrcaER) using Metamorph software. Images were taken every 15 s with an exposure of 200–1000 ms.

Authors' contributions
JY prepared the manuscript and performed the time-lapse microscopy, *Listeria*, and myosin ATPase experiments. Remaining experimentswere done in collaboration with TL. RL and TM supervised this work and provided funding and support. All authors read and approved the final manuscript.

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