Synthetic polyamines: new compounds specific to actin dynamics for mammalian cell and fission yeast

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Abbreviations: ADF, Actin depolymerizing factor; BPA, Branched polyamines; EMM5S, Edinburgh Minimal Media with 5 Supplements; F-actin, Filamentous actin; GFP, Green fluorescent protein; MPA, Macrocyclic polyamines; N-WASP, neuronal Wiskott-Aldrich syndrome protein; Rlc1, Regulatory light chain 1; ROCK, Rho-associated protein kinase.

Actin is a major actor in the determination of cell shape. On the one hand, site-directed assembly/disassembly cycles of actin filaments drive protrusive force leading to lamellipodia and filopodia dynamics. Force produced by actin similarly contributes in membrane scission in endocytosis or Golgi remodeling. On the other hand, cellular processes like adhesion, immune synapse, cortex dynamics or cytokinesis are achieved by combining acto-myosin contractility and actin assembly in a complex and not fully understood manner. New chemical compounds are therefore needed to disentangle acto-myosin and actin dynamics. We have found that synthetic, cell permeant, short polyamines are promising new actin regulators in this context. They generate growth and stabilization of lamellipodia within minutes by slowing down the actin assembly/disassembly cycle and facilitating nucleation. We now report that these polyamines also slow down cytokinetic ring closure in fission yeast. This shows that these synthetic compounds are active also in yeasts, and these experiments specifically highlight that actin depolymerization is involved in the ring closure. Thus, synthetic polyamines appear to be potentially powerful agents in a quantitative approach to the role of actin in complex processes in cell biology, developmental biology and potentially cancer research.

Acto-Myosin and Actin Dynamics as Key Players of Cell Dynamics

Cells and tissues change their shapes through 2 basic molecular mechanisms. Myosin motors interact with actin filaments and they can by themselves generate forces in a variety of phenomena, ranging from muscle contraction and stress fibers dynamics to intracellular transport processes. On the other hand, actin cycles of polymerization and depolymerization are also dictating morphogenesis events in the absence of myosin, like in the dynamics of membrane protrusions with filopodia and lamellipodia in mammalian cells. In these examples, each machinery can sustain forces and deformations in the absence of the other machinery. Indeed, it was shown experimentally^{1,2} and theoretically^{3,4} that both acto-myosin interactions and actin dynamics alone can generate stresses and strains.

However both force-producing machineries are often coupled in cells and tissues, and it is difficult to discriminate the specific contribution of each in the global process. For example, in the cytokinetic ring, bundles of actin and myosins are organized in a ring which undergoes closure. It was suggested

that actin polymerization was a key player in these processes by experiments with mutants in actin dynamics leading to a delay in the ring closure of fission yeast.⁵ On the other hand, experiments with spheroplasts - rings in cellular fragments extracted from fission yeasts - showed that myosin was mainly piloting the closure through its interactions with actin filaments.⁶ Similar results were obtained in actin rings assembled *in vitro*.⁷

The interplay between the above force-producing machineries actually seems to be effective in most motile and morphogenetic processes. Cell migration requires a balance between actin-based protrusion and acto-myosin-driven contractility.⁸ Adherens junctions maturation was reported to involve an interplay between acto-myosin forces at the junctions together with actin polymerization in the neighboring cell.^{9,10} In the highly spatially organized podosome structures, the dynamics of protrusive cores and adhesive rings is coordinated by acto-myosin.¹¹ In developmental biology too, both machineries were demonstrated to be acting in concert. For example, cell division in *Drosophila* involves the action of myosin under the control of ROCK as well as actin

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polymerization for keeping the tissue integrity between neighboring cells.¹²

A potential way to understand the molecular basis of these phenomena is to consider that both machineries cooperate. The acto-myosin could be pulling cellular structures which in turn will allow the insertion of actin monomers in a new regime of polymerization/depolymerization cycles. In support of this view, applying a pulling force to formin-initiated filaments in processive elongation increases their growth rate.¹³ Similarly, growth of focal contacts results from the force applied by contractile stress fibers on focal adhesions, and the elongation of focal contacts requires the polymerization of actin.¹⁴ In such a framework, there is a need for new specific tools to evaluate the contributions of each actor in the process.

Limited Set of Chemical Agents Acting on Actin

A limited set of agents are available (see Table 1), and they often lead to the disassembly of cellular structures. For example, Latrunculin A sequesters monomeric actin, causing disassembly of all actin meshworks in protrusive lamellipodia and contractile structures. Cytochalasins, as well as a large number of macrolides derived from marine organisms¹⁵⁻¹⁷ generally strongly cap the barbed ends of actin filaments and often also sever filaments and trigger the disassembly of cellular structures. These agents are widely used to demonstrate that actin is involved in the process of interest, and they are complementary to transfection or genetic approaches which target a fraction of cells within tissues. Less brutal force tools, however, are required to gain mechanistic insight into the exact role of acto-myosin contractility or actin assembly in a global process.

Synthetic Polyamines as New Agents with Broad Application

We observed that addition of synthetic polyamines to cell culture medium promotes the growth of lamellipodia in cells within a few minutes.¹⁸ Their direct and specific effect on actin was demonstrated by *in vivo* experiments with inhibitors and *in vitro* actin polymerization assays. We have shown that the compounds slow down filament elongation at barbed ends specifically, but do not affect pointed end growth. They also slow down filament disassembly in a concentration-dependent manner, and facilitate nucleation in the presence of capping protein.

Figure 1. The compounds C₇N₆ MPA (A) and C₈N₆ BPA (B). The cyclic and branched polyamines have similar effects even though their topologies are different.

The Compounds, their Structures, and the Active Groups

Macrocyclic polyamines have been shown to greatly enhance actin polymerization *in vitro*.¹⁹ Based on these structures, non-cyclic branched polyamines were designed and synthesized for exploration of their effect on actin.^{18,20} Both cyclic and branched polyamines of different sizes affected actin assembly similarly with affinities that depended on their size. Here we present 2 compounds that proved the most efficient ones in our study: (i) macrocyclic polyamines C₇N₆ MPA (MPAs), presenting 2 dipropylene triamine subunits linked by 2 aliphatic 7-carbon chains (Fig. 1A), and (ii) acyclic branched polyamines C₈N₆ BPA (BPAs), 2 dipropylene triamine groups connected through the central nitrogen by an octamethylene chain (Fig. 1B). In spite of their distinct topologies, MPAs and BPAs display identical effects on actin *in vitro* and *in vivo*, which suggests that it is essentially the distance between amine groups which play an important role in their activities.

Growth of Lamellipodia: De Novo Extension with Formation of Focal Contacts

We observed that following rapid entry of the compounds into the cell, lamellipodia were growing within 10 minutes in a non-polarized fashion, from various regions at the edge of the cells (see Fig. 2 and Movie 1). We show here the local growth of lamellipodia observed with actin-GFP transfected cells (Movie 1). They were *bona fide* lamellipodia, in a sense that focal contacts were associated with the growing protrusions (Fig. 2C). Other experiments in the presence of inhibitors of the Rho pathway and actin polymerization showed that actin was the main target of MPAs/BPAs.¹⁸

Table 1. Standard compounds targeted to the actin cytoskeleton and their effects. For more details, see associated references in¹⁸.

Compounds	Effects
Synthetic Polyamines	slow down F-actin turn-over at the barbed end and facilitate F-actin nucleation
Cytochalasin D	inhibits actin polymerisation by binding filament barbed end at low concentration (<0.1 μM) and promotes depolymerisation at high concentration (>1 μM)
Jasplakinolide	binds and stabilises F-actin and blocks filament disassembly
Latrunculin A/B	sequester monomeric actin and promote actin depolymerisation
CK666	inhibits Arp2/3
SMIFH2	inhibits formins
Wiskostatin	inhibits N-WASP

Correlations between *In Vivo* and *In Vitro* Effects of Synthetic Polyamines

We gained insight into the mechanism by studying the dynamics of actin polymerisation in various conditions *in vitro*. We showed that the compounds slowed down barbed end assembly specifically and had no effect on pointed end growth. They also slow down filament disassembly and greatly depress the effect of ADF. Consistently in a reconstituted motility assays, N-WASP coated beads move more slowly. The slower turn-over of actin filaments in the motility assay suggested that *in vivo* lamellipodial actin networks should also turn-over more slowly in the presence of polyamines, which was confirmed by measuring retrograde flow in cells.¹⁸

Finally, we demonstrated that actin nucleation was facilitated *in vitro* in the presence of capping proteins. So the molecular picture *in vivo* combines 2 effects: the stabilization of the lamellipodia over time together with the facilitated growth of the actin network at the cell border. Note that the absence of filopodia is consistent with the known facilitation, by capping protein, of dendritic branched filament arrays present in lamellipodia versus formin-initiated filament bundles present in filopodia, both *in vivo*²¹ and *in vitro*.²²

Synthetic Polyamines Slow Down Cytokinetic Ring Closure

We tested whether MPAs and BPAs would similarly affect actin dynamics in another eukaryotic cell. We selected fission yeast as a powerful genetic system. In addition, we focused on cytokinetic ring closure, because it was suggested that actin dynamics could play a role in the velocity of closure.⁵

We observed the ring with a strain expressing myosin II regulatory light chain Rlc1-tdTomato. We measured the change in

Figure 2. Synthetic polyamines promote growth of lamellipodia. (A) Promoted growth of lamellipodia in NIH3T3 cells; scale bar 30 μ m; (B) Dynamics of the growth visualized with actin-GFP in NIH3T3 cells, after addition of 300 μ M C₇N₆ MPA (see also **Movie 1** and ref.¹⁸); time in minutes; arrows indicate new lamellipodia; scale bar 5 μ m. (C) Focal contacts are associated with new lamellipodia (actin in green and paxillin in red); scale bar 20 μ m.

diameter as a function of time during the ring closure (see **Fig. 3** and **Movie 2**). We observed that ring closure was slowed down on average by 50% in the presence of 300 μ M C₇N₆ MPA, and by 20% and 40% in the presence of 100 μ M and 300 μ M C₈N₆

Figure 3. Synthetic polyamines delay the closure of the cytokinetic ring in fission yeast. (A) Fission yeast at cytokinesis, (left) phase contrast, (right) the ring visualized with Rlc1-tdTomato, scale bar 2 μ m; (B) Montage of dynamics of the ring diameter during closure for control cells and in the presence of C₇N₆ MPA; images were equalized in their contrast; scale bar 4 μ m. (C) Mean velocities for control rings (66 cells) and in the presence of 300 μ M C₇N₆ MPA (76 cells). The error bars represent the standard deviation of the mean. $P \leq 0.01$ by Student's t-test. See also **Movie 2**.

Movie 1 Growth of lamellipodia promoted by C₇N₆ MPA in a NIH3T3 cell transfected with actin-GFP, time in mm:ss, scale bar 5 μ m.

BPA respectively (data not shown). This result is consistent with former experiments with mutants in which actin filament turnover was slowed down during cytokinesis.⁵

Future experiments with mutants in actin polymerization will probe the specific mechanisms that lead to the slowed closure and the relative contributions of actin filament disassembly and acto-myosin contractility at different steps of ring closure. A possible interpretation consistent with *in vitro* data is that MPA and BPA slow down the ADF-induced depolymerization that has been proposed to take place during ring closure, accounting for the decrease in amount of F-actin, at least in budding yeast.²³ Here we can speculate about a potential origin - knowing that MPA/BPAs have no effects on acto-myosin activities: these compounds could be binding actin filaments within the cytokinetic ring, slowing down depolymerization and treadmilling, thus slowing down the decrease in ring diameter. The facilitated nucleation might also generate new filaments within the closing ring, thereby impeding the reduction in amount and diameter.

New Compounds for the Study of Actin

We have shown that MPA and BPA can be good probes targeting the actin cytoskeleton in mammalian cells and in fission yeast. We explored the *de novo* growth of lamellipodia and the contraction of the cytokinetic ring. We anticipate that other cellular processes could be studied as well, such as endocytosis, transport mediated by actin polymerization, dynamics of the actin cytoskeleton through nodes, cell motility. In addition, other model organisms could be probed as well, such as budding yeast or developing embryos. Synthetic polyamines, by altering specific steps in shape changes of developing embryos, could provide insight into the underlying molecular and physical mechanisms.

Application in Cancer: Reduced Motility and Delayed Division

Actin is involved in a variety of phenomena, and a potential application in medicine could be cancer. Indeed, actin plays a key role in motility and in cell division: we propose that BPAs/MPAs would lead to a decrease in cell motility (see our paper¹⁸ for the decreased migration in Boyden chamber) and to a delay in the proliferation rate. Also, it was recently shown that BPAs have a strong inhibitory effect on the transformation of cancer-

associated fibroblasts into myofibroblasts, a key step in tumor progression.²⁴ Anti-actin compounds have failed so far as anti-cancer agents, because of the poor discrimination between actin in cancer cells and in healthy cells.²⁵ Hence, careful experiments on the small animal will be needed to evaluate the cytotoxicity of the BPAs/MPAs and their potential specific effects on tumors.

Methods

The MPAs and BPAs were synthesized in the Lehn Lab. C₈N₆ BPA can now be purchased from Tebu and Calbiochem. For mammalian cells (**Fig. 2B**), NIH3T3 cells were transfected with actin-GFP with Exgen using the manufacturer protocol (Euromedex). The other examples for mammalian cells (NIH3T3 cells) are presented in the original publication. For fission yeast experiments, cells were grown in standard exponential conditions.²⁶ Cells were deposited on coverslips coated with lectin,²⁷ and experiments were conducted in minimal medium EMM5S at 25°C in a temperature controlled environment. Rings were captured with a ×60 objective 1.45 N.A. mounted on an inverted CKX41 microscope (Olympus) equipped with a cooled couple-charge device camera (C4742–96–12G04; Hamamatsu). Each experimental condition was performed at least for 5 biological repeats. Rings were selected for their 'large' diameters (2.5 μm–3 μm) at the beginning of closure, and each ring was acquired every 60s with a 100ms or 200ms time exposure. Measurements were performed with ImageJ, and the evolution of diameter and measurements for velocities were done with Excel.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental data for this article can be accessed on the publisher's website.

Movie 2 Closure of the cytokinetic ring in fission yeast visualized with Rlc1-tdTomato, time in mm:ss, comparison between control (left), and in the presence of C₇N₆ MPA (right). The frames were equalized in their contrast. Time in mm:ss, scale bar 2 μm.

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