Chivosazole A modulates protein-protein-interactions of actin

Shuaijun Wang†, Florian A. Gegenfurtner‡, Alvaro H. Crevenna§, Christoph Ziegenhain‡, Zane Kliesmete§, Wolfgang Enard§, Rolf Müller┴, Angelika M. Vollmar‡, Sabine Schneider∇*# and Stefan Zahler†*#

†Department of Pharmacy, Ludwig-Maximilians- University, 81377 Munich, Germany
‡Biomolecular Self-Organization Laboratory, ITQB-Universidade Nova de Lisboa, 2780-157 Oeiras, Portugal
§Department of Biology II, Ludwig-Maximilians-University, 82152 Planegg-Martinsried, Germany
┴Department of Pharmacy, Saarland University, 66125 Saarbrücken, Germany
¶Department of Microbial Natural Products, Helmholtz Institute for Pharmaceutical Research Saarland and Helmholtz Centre for Infection Research, Saarbrücken, Germany.
∇Department of Chemistry, Technical University Munich, 85748 Garching, Germany
*Correspondence (structural biology): sabine.schneider@mytum.de
*Correspondence (cell biology and biochemistry): stefan.zahler@cup.uni-muenchen.de, Tel. ++4989218077196
#These authors contributed equally
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ABSTRACT Actin is a protein of central importance for many cellular key processes. It is regulated by local interactions with a large number of actin binding proteins (ABPs). Various compounds are known either increasing or decreasing polymerization dynamics of actin. However, no actin binding compound has been developed for clinical applications yet, due to selectivity issues. We provide a crystal structure of the natural product chivosazole A (ChivoA) bound to actin, and show that – in addition to inhibiting nucleation, polymerization and severing of F-actin filaments – it selectively modulates binding of ABPs to G-actin: While unphysiological actin dimers are induced by ChivoA, interaction with gelsolin, profilin, coflin and thymosin-beta4 is inhibited. Moreover, ChivoA causes transcriptional effects differing from latrunculin B, an actin binder with a different binding site. Our data show that ChivoA and related compounds could serve as scaffolds for the development of actin binding molecules selectively targeting specific actin functions.
Actin is the most abundant protein in eukaryotic cells, and is indispensable for many cellular processes, such as migration, maintenance of cell shape, cell division, or transport of vesicles, due to its ability to form filaments that integrate into crosslinked networks in the cytoplasm of cells. Furthermore, actin is the protein with the most known protein-protein-interactions (1). The best studied interaction of actin monomers is the one with other actin monomers in the process of nucleation and elongation of F-actin fibers, which is locally regulated by actin binding proteins (nucleators, stabilizers, capping or severing proteins). In addition, actin is an integral part of many protein complexes, such as those that regulate transcription, DNA damage repair, or chromatin remodeling (2-4). In addition, the actin cytoskeleton and actin polymerization dynamics are altered in malignant cells, as a result of the activation of oncogenic actin signaling pathways (e.g., Ras), or the inactivation of actin-binding proteins with tumor suppressor functions (e.g., gelsolin). Thus actin remodeling not only determines the morphology and phenotype of cancer cells, but is directly linked to the malignant transformation process and metastasis (5).

This functional versatility makes actin a fascinating molecule to study, but also limits the use of actin binding compounds as lead structures for drug development due to its intricate involvement in many essential cellular processes. Though actin binding natural compounds are known for more than 45 years (6), none of them has made it into the clinic due to lack of functional selectivity. The large number of actin binding compounds we know today is mainly of natural origin (fungi, bacteria, marine organisms) and can be roughly divided into two groups: 1) actin filament stabilizers, and 2) filament assembly inhibitors or destabilizers (7). Interestingly, the binding sites of most of these compounds are located at the hydrophobic plug or the hydrophobic cleft at the barbed end of G-actin, where most actin binding proteins also interact (1, 7). The
concept has been developed that macrolide toxins are able to mimic the binding of actin capping proteins to actin (8, 9). It has recently been hypothesized that the local functional specificity of actin originates from local interactions with actin binding proteins, based on competition (10). Consequently, it would be feasible to change these local competitive interactions by using respective small molecules, thus affording specificity of an actin binding molecule towards a defined function of actin.

In the present work we characterize the actin binding mode of ChivoA, a macrolide of myxobacterial origin (11) with actin filament inhibiting properties (12), through co-crystallization and structure determination. In addition we investigate its effects on actin polymerization, protein-protein-interactions of actin, cellular function, and transcriptional regulation in comparison to LatB, a structurally unrelated actin filament inhibitor. In summary, our data provide penetrating insights into the molecular mechanisms of actin cytoskeleton remodeling and resulting impact on gene regulation by the natural product ChivoA. Moreover, we show that ChivoA and related compounds could serve as scaffolds for the development of molecules to target specific actin functions.
RESULTS AND DISCUSSION

Crystallisation and structure determination of G-actin in complex with ChivoA: ChivoA was initially described as cytotoxic agent in eukaryotic cells in 1995 (11), and its absolute configuration was resolved 12 years later (13). Its actin interfering effects (inhibition of G-actin polymerization and depolymerisation of F-actin) were identified in 2009 (12). One could argue that ChivoA is just another of many known natural compounds, that interferes with actin polymerisation dynamics (7). However, each of these compounds has unique functional features and it is worthwhile to study their structure-activity-relationships. Very recently, chivosazole F (ChivoF), a compound closely related to ChivoA, has been shown to directly interact with actin by chemoproteomics (14). Based on genome-wide mutagenesis studies in yeast with ChivoF, it was proposed that ChivoA would bind close to the binding site of latrunculin A (14). In order to elucidate the binding mode of ChivoA to actin, we first determined the X-ray crystal structure of ChivoA in complex with G-actin to 2.4 Å resolution. (Fig. 1A) (for data collection and structure refinement statistics see Table S1). ChivoA binds to the barbed end (target binding cleft) of G-actin (Fig. 1B and Fig. S1), as described for a number of other, stereochemically diverse macrolides and actin binding proteins (15-24), (Fig. 1C). The discrepancy between the previously suggested binding site and our finding could be explained by the fact that we used ChivoA, while ChivoF was used in the other work. However, this seems unlikely, as both compounds are nearly identical (Fig. 1 and Fig. S3). It is feasible that the sequence of actin (which is evolutionary highly conserved) lacks flexibility, and mutations at the real binding site do not yield functional protein. The described mutations (R183K and R335K) might have allosteric consequences, influencing binding of ChivoA at a distant site. The binding site we describe for ChivoA is very close, but not identical to those of many actin binding macrolides.
Despite the variation in ring size and decoration of the tail with different backbone substitutions present in actin-binding macrolides, they possess a common theme in their interaction with actin. In all reported actin complex-structures, the macrolactone ring binds to subdomain 1 with the side chain/tail protruding into the hydrophobic cavity between subdomain 1 and 3 (8, 15-18, 22, 24-26). The oxazole ring of ChivoA occupies an intermediate position compared to the trisoxazole rings of jaspisamide A, kabiramide C and ulapulaide A (8, 24) (Fig. 1C). Albeit we cannot rule out that crystal lattice contacts impact the binding mode of ChivoA (Fig. S1), for jaspisamide A and kabiramide C identical interactions with actin were observed in structures obtained from different crystal symmetries. Interestingly, the binding site of ChivoA overlaps with that for a number of actin-binding proteins (ABPs). This might explain, why ChivoA competes with coflin, gelsolin, profilin or thymosin-β4 for binding to G-actin, while LatB does not. These proteins interact with actin by insertion of an amphiphilic helix into the target binding cleft as depicted in Fig. S4 (19-21, 23, 27). Recently the cryo-EM structure of F-actin has been reported (PDB code 6BNO (28)), revealing an interface of 1,076 Å² between the actin-monomers (theoretical gain of free energy of -6.9 kcal/mol). Here the D-loop (residue 40-50) slides into the target binding cleft between subdomain 1 and 3. Superposition of the F-actin structure and the actin-ChivoA complex shows that the ChivoA-tail partly occupies the binding site for the DNase-binding loop of the neighbouring actin monomer, and, in addition, slightly perturbs other structural elements involved in actin-actin interaction (Fig. 1D). This provides the structural basis for the actin-depolymerizing action of ChivoA.
Figure 1. X-ray crystal structure of G-actin in complex with ChivoA. A) Chemical structure of ChivoA. B) Structure of the actin-ChivoA complex, overall folding topology and domain structure. Actin is depicted as ribbon, with the four domains highlighted. The ATP (orange) and ChivoA (green) are shown as stick model, the magnesium ion as sphere (purple). Overlaid, for ChivoA the simulated annealing Fo-DFc difference electron density is contoured at 2.5 σ (green). C) Superposition of actin structures in complex with macrolides. The actin surface is colored according to the surface residue charges (blue=positive, red =negative) and the macrolides are shown as stick models. ChivoA (dark green, this work), lobophorolide (light green, PDB code 3M6G), jasplakinolide A (cyan; PDB code 1QZ6) and sphinxolide B (gold;
ChivoA sequesters G-actin, inhibits actin nucleation and polymerization and destabilizes F-actin in vitro. To characterize the functional effects of ChivoA we performed bulk assays with pyrene labelled actin and TIRF assays with Atto488 labelled actin. For comparison we used the prototypic actin depolymerizer latrunculin B (LatB), which has a different binding site and mode of action (15): it binds to the ATP binding pocket between domains 2 and 4 of actin, thus inhibiting ATP breakdown. ChivoA dose dependently inhibits actin polymerization (Fig. 2A), as previously described by others (12) with a somewhat lower potency that LatB. After an initial inhibition of polymerization, depolymerisation (drop of fluorescence intensity) becomes also visible at a later time point at higher concentrations of ChivoA. When investigating the underlying mechanisms, we found that the critical concentration of actin in the presence of either LatB or ChivoA, was increased to a similar degree (Fig. 2B). This indicates a similar sequestering action of both compounds. Accordingly, we found a decreased nucleation of actin in TIRF assays (Fig. 2C), and a lower rate of polymerization (Fig. 2D). In addition, we observed a destabilizing effect of ChivoA on F-actin filaments (Fig. 2E).
Figure 2. ChivoA sequesters G-actin, inhibits actin nucleation and polymerization and destabilizes F-actin in vitro. A) ChivoA and LatB both concentration dependently inhibit
polymerization of pyrene actin. Maximum fluorescence intensity of the control was defined as 100% and the other fluorescence intensities normalized accordingly. B) 2 µM of LatB (positive control) and ChivoA shift the critical concentration of actin for polymerization to a similar degree. C and D) In TIRF assays, ChivoA inhibits nucleation of actin and the elongation rate. The number of filaments and the growth rate in control experiments was set as 1.0 and the other values were normalized against controls. E) F-actin filaments are destabilized by ChivoA in the TIRF assay. Data are presented as mean ± SEM, n = 3, *p < 0.05 using Kruskal-Wallis test.

ChivoA inhibits proliferation and migration, and changes actin architecture in endothelial cells. In order to get insights into potential functional differences between ChivoA and LatB (in spite of their similar actions on actin alone), we performed comparative experiments on a cellular level. In human primary endothelial cells (HUVECs) ChivoA inhibited proliferation with an IC_{50} of approx. 3 nM. This is in good accordance with previously published values in different mammalian cells (12), and demonstrates ChivoA to be much more potent than LatB (Fig. 3A). Interestingly, LatB was the more potent compound in the pure in vitro assays. This might be due to either a higher metabolic stability of ChivoA in comparison to LatB in cells or to a better membrane permeability by ChivoA. Morphologically, ChivoA causes an overall loss of F-actin fibers, which is not restricted to subcellular structures like filopodia or lamellipodia. At the same time amorphous actin aggregates occur with increasing concentration (Fig. 3B). We cannot distinguish, whether these are fragments of contracted F-actin bundles, or aggregates of sequestered G-actin. Since cellular motility is a central aspect of actin function, we investigated the effects of the two compounds on migration of HUVECs in a scratch assay and specifically on directional migration (chemotaxis). The IC_{50} value concerning migration was 10-fold higher
compared to proliferation (Fig. 3C). This is most likely due to the shorter duration of the migration assay (16 h vs. 72 h). In the chemotaxis assay both, overall features of cell motility (velocity), and directional components of migration were assessed. Both compounds caused a decrease of cell velocity, and, additionally, a loss of the “sense of direction” (Fig. S2).

**Figure 3.** ChivoA inhibits proliferation and migration, and changes actin architecture in endothelial cells. A) ChivoA (left panel) inhibits the proliferation of primary human endothelial cells much more potently than LatB (right panel). Proliferation of controls was defined as 100% and the other experiments were normalized to the respective control. B) The actin cytoskeleton
of HUVECs is rapidly disrupted by ChivoA. Lower panel: Zoom in of the white frames indicated as boxes in the upper panels. Blue: nuclei stained with Hoechst, red: F-actin stained with rhodamine-phalloidin. Scale bars: 25 µm (upper panel), 10 µm (lower panel). C) Upper panel: Dose response curves of cell migration after treatment with ChivoA or LatB in a scratch assay (lower panel: representative images of scratches after migration). The cell covered area in the scratch region was quantified and expressed as %. Scale bar: 200 µm. Data are presented as mean ± SEM, n = 3. See also Figure S3.

ChivoA and LatB affect transcriptional regulation in a different manner. As the two compounds showed no obvious differences concerning their actions on general parameters like proliferation or migration, we chose an unbiased transcriptional approach for their further functional comparison and investigated the effects of subtoxic doses of ChivoA and LatB on gene expression in endothelial cells. Both compounds led to marked changes in transcription: 62 genes were downregulated and 39 genes were upregulated after treatment with ChivoA, 27 genes were downregulated and 62 upregulated with LatB (Tables S2 and S3). Of these genes, 29 protein coding genes were only downregulated by ChivoA, but not by LatB, and 19 were only upregulated by ChivoA and not by LatB. 5 genes were specifically downregulated and 40 upregulated by LatB in contrast to ChivoA (Table S4).

ChivoA competes with actin binding proteins for binding to G-actin and causes dimerization of actin: Based on the surprising functional differences between the two compounds concerning transcription, and the fact that actin influences transcriptional regulation mainly by its interaction with ABPs (29), we tested the effects of the two compounds on the binding of
several proteins (cofilin, gelsolin and profilin) to G-actin. We performed an actin pull-down assay with G-actin immobilized on beads and single ABPs in the absence and presence of ChivoA or LatB, then quantified the ABP binding to G-actin. As seen in Fig. 4A-C (left panels), the total amount of the tested ABPs in G-actin pellets decreased with ChivoA (molar ratio 1:100), indicating that it prevented binding of cofilin, gelsolin and profilin to G-actin. In contrast, LatB had no such effect (Fig. 4A-C, right panels), which offers a potential explanation for the functional differences between these two compounds. However, this is only speculative especially in the light of the much higher concentrations of ChivoA needed for the in vitro competition with ABPs in comparison to the cellular effects.
Figure 4. ChivoA prevents the binding of proteins (ABPs) to G-actin, while Lat B does not. G-actin beads were pretreated with ChivoA and LatB respectively for 30 min and then co-incubated with the actin binding proteins cofilin (A), gelsolin (B) or profilin (C) at a molar ratio of 100:1 (compound:ABP). After 1 h, the mixture of actin beads and ligands was spun and only the ligands bound to G-actin will be co-precipitated in the pellet. The amount of ABP in pellet was quantified. Representative images of protein bands are shown. Data are presented as mean ± SEM, n = 3, *p<0.05, **p<0.01 vs. control (Kruskal-Wallis test with Dunns test as post hoc).
The gray values of the bands in the pellet fraction of each protein in untreated controls was defined as 1.0 and the other bands normalized accordingly.

Since the binding affinity of thymosin β4 is very weak, and we were not able to detect this protein in the pulldown assay, we crosslinked the bound protein as previously described (30). With crosslinking, a 1:1 complex of thymosin β4 with G-actin at approximately 47.5 kDa is detectable (Fig. 5A, left panel). ChivoA reduced crosslink product formation (Fig. 5A, right panel). In addition, a high molecular weight complex (approximately 100 kDa) was formed in the presence of ChivoA (Fig. 5A, left panel and also in the absence of thymosin, Fig. 5C), suggesting the formation of G-actin dimers. In some experiments, a further band at approx. 120 kDa occurred (Fig. 5A), which can not be explained currently: it is too small for actin trimers and too large for a ternary complex of thymosin with actin dimers. LatB decreased the formation of the thymosin β4/G-actin complex (Fig. 5B), but did not cause formation of actin dimers. Gel filtration experiments also showed an increase of actin dimers in the presence of ChivoA under conditions of a different pH (Fig. 5D). However, the retention time of these dimers differs from that of spontaneously formed physiological dimers, indicating a different overall shape (Fig. 5D). ChivoF (Fig. 3S), a derivative which is structurally very similar to ChivoA, and rhizopodin, an also structurally related compound, inhibited binding of profilin, gelsolin and cofilin to a similar degree as Chivo A (Fig. S3). The occurrence of actin dimers has already been described with rhizopodin and swinholide A (25, 26) compounds which possess a two-fold symmetry with two enamide side chains each binding one actin molecule resulting in a one-to-two complex. The symmetry of the macrolides explains the actin dimerization observed in these complex crystal structures. However, both studies state that due to absence of a stable interaction interface between the two actin molecules, the observed dimers are non-physiological (25, 26). In contrast,
ChivoA possess only one side chain, with one ChivoA-molecule binding one actin monomer (Fig. 1). Crystal contacts between the actin-ChivoA complexes occur between the two adjacent CivoA molecules (see above and Fig. S1B). Neighboring actin molecules in the crystal lattice contact do not form stable interfaces (PISA analysis). Previously reported crosslinking studies on actin-kabramide C (KabC) complexes followed by crystallisation resulted in laterally crosslinked dimers related by the 2(1) symmetry of the crystal lattice (31), which was also observed in a previous study by Dawson et al. (32). The authors argue while this may reflect a possible mode of actin-actin interaction, it does not resemble the native filamentous form of actin. However, in the here reported actin-ChivoA complex structure the assembly of the crystal lattice is different to the laterally cross linked actin-KabC complexes. Thus albeit we obtained detailed structural information on the molecular interactions between ChivoA and actin, we cannot deduce the possible ChivoA-induced dimer interactions/interface. Nevertheless, the binding affinity needs to be in the low micro molar range since the dimers are stable during size-exclusion chromatography. Due to the altered SEC-elution profile of the ChivoA-actin dimers, one might speculate that actin dimers in the presence of ChivoA could resemble the lateral crosslinked form, while actin-dimers in the absence of ChivoA could form dimers similar to the longitudinal form found in F-actin. It has recently been shown that the induction of unphysiological actin oligomers by toxins can cause dramatically altered affinity towards ABPs (33). This effect could further contribute to the higher efficacy of ChivoA in comparison to LatB.
Figure 5. ChivoA prevents binding of thymosin β4 to G-actin and causes formation of unphysiological actin dimers. (A) Binding of thymosin β4 (4 µM) to G-actin could only be
detected after crosslinking. This interaction was inhibited by ChivoA (40 µM). In the presence of ChivoA actin dimers formed. (B) Binding of thymosin β4 to G-actin was also inhibited by 40 µM LatB, however, no actin dimers were formed in the presence of this compound. The gray values of the bands of the thymosin crosslinked to actin in untreated controls was defined as 1.0 and the other bands normalized accordingly. (C) The actin dimers formed by ChivoA were not dependent on the presence of thymosin β4. Representative images of protein bands and quantitative densitometric analyses are shown (**p<0.01 vs. control, Kruskal-Wallis test with Dunn’s test as post hoc, n=3). (D) Size-exclusion chromatography at different pH values indicate the formation of actin-dimers in the presence of ChivoA. Dimers formed spontaneously in the absence of ChivoA elute at a time point different from the drug induced ones, hinting at an unphysiological conformation of the latter ones.

Consequently, this reveals a previously unexpected layer of complexity in the mechanism of action of actin binding compounds: instead of just causing bulk stabilization or de-stabilization of actin fibers, distinct cellular functions could be preferentially addressed by single compounds. “Biomolecular mimicry” (9), the fact that compounds can selectively compete with ABPs, and the high structural diversity of actin binding compounds might team up to generate an unprecedented functional selectivity for pharmacologically targeting actin. Since the total synthesis of ChivoF has been resolved (34, 35), and chivosazoles can be produced biotechnologically, these compounds might be ideal candidates for derivatization and for exploring structure-activity-relationships with actin.
Experimental Section

Compounds

ChivoA, ChivoF, and rhizopodin were isolated from myxobacterial strains as previously reported (11, 36). LatB was purchased from Sigma-Aldrich (Taufkirchen, Germany).

Crystallization and structure determination of G-actin in complex with ChivoA

Lyophilised rabbit α-actin (Hypermol) was dissolved in 2 mM hydroxyethyl piperazineethanesulfonic acid (HEPES-Na), 0.2 mM adenosine triphosphate, 0.2 mM calcium chloride, 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to a concentration of 2 mg/ml, mixed with 1.5 equimolar ChivoA (from 10 mM stock in dimethyl sulfoxide) and incubated for 30 min on ice (final α-actin concentration 1 mg/ml). The protein was concentrated to 10 mg/ml using centrifugal filter devices (Millipore), centrifuged to remove debris (15 min, 16000 g, 4°C) and high-throughput crystallisation screening was carried out (Phoenix, Art Robbins; Nextal DW Block Suits, Qiagen). Crystals of G-actin in complex with ChivoA were obtained by the sitting-drop vapour diffusion method, in 0.5 M lithium chloride, 100 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 28 % (w/v) polyethyleneglycol 6000, pH 8.5, at 20°C. Prior to cryo-cooling and storage in liquid nitrogen, crystals were dipped into reservoir solution supplemented with 20% (w/v) ethyleneglycol for cryo-protection. Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF, ID23-2), Grenoble, France. The data were processed with XDS (37) and the crystals belonged to the space group P2₁. The resolution cut-offs were chosen according to the correlation coefficient of random half-data sets (CC 1/2) at about 50% (38-40). The structure was solved using the coordinates of a previously reported structure (PDB code 1YXQ) by molecular
replacement in PHASER. Following simulated annealing with PHENIX, clear peaks for ChivoA were visible in the Fo-Fc difference electron density (Supplemental Figure 1). This was followed by iterative cycles of manual model building in COOT and restraint and TLS refinement in REFMAC5. Structure optimisation was carried out using the PDBredo server. For data processing and structure refinement statistics see Table S1. Interaction interfaces were analysed using PISA. All structural figures were prepared with PyMol (Delano Scientific). A schematic diagram of the interactions between actin and ChivoA was prepared with LigPlot.

*Pyrene assay*

10% pyrene actin from rabbit skeletal muscle was purchased from Hypermol. Pyrene actin was diluted with H$_2$O to obtain a stock solution of 1mg/mL (24 μM) and subsequently centrifuged at 100,000 x g at 4°C for 1 h to remove any actin aggregates. 50 μL of actin polymerization solution consisted of: 30 μL H$_2$O, 10 μL 10 mM MgCl$_2$, 5 μL F-actin buffer (100 mM imidazole-Cl, 10 mM ATP, pH 7.4), as well as 5 μL of either ChivoA or DMSO (solvent control). Actin polymerization was started by rapid addition of 10 μL of pyrene actin. Pyrene fluorescence was monitored every 20 s over 1 h in a 96-well fluorescence plate reader at 360 nm excitation and 400 nm emission.

For the determination of the critical concentration of actin, pyrene actin at concentrations from 200 nM to 2 μM was incubated with either 2 μM ChivoA or LatB as indicated. After 24 h the final pyrene fluorescence was determined.
**TIRF assay**

Flow cells (containing 15 - 20 μL of fluid) were prepared as a sandwich of a cover slip (22 x 22 mm), two parafilm strips separated by a 5 mm gap and a glass slide (76 x 26 mm). Solutions were loaded directly into the chamber via capillary action. Labeled actin was prepared by mixing Atto488-actin (Hypermol) and actin from rabbit skeletal muscle (Hypermol) 1:1 (v/v). α-actinin from turkey gizzard smooth muscle (Hypermol, Bielefeld, Germany) was dissolved in ultrapure water to obtain a 1 mg/mL stock solution, and used as tethering protein.

**Nucleation and polymerization assay**

The flow cell was passivated by incubation it with 25 μL 1% (w/v) of bovine serum albumin (BSA) in phosphor buffered saline (PBS) for 10 min. 25 μL of tethering protein α-actnin (1 mg/mL) was applied for 5 min. Labeled actin (10 μM) was incubated 1:1 with 1/10 volume of 10 x Mg-exchange buffer (2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 400 μM MgCl₂) and 1:8 with G-buffer (2 mM Tris-HCl, pH 8.0, 0.2 mM CaCl₂, 0.5 mM dithiothreitol (DTT), 0.2 mM ATP, final pH 7.8) on ice for 5 min to exchange Ca-ATP-actin for Mg-ATP-actin. Polymerization was triggered by mixing Mg-ATP-actin with 2 x TIRF buffer (100 mM KCl, 2 mM MgCl₂, 2 mM EGTA, 30 mM imidazole, 30 mM D-glucose, 40 μg/mL catalase, 400 μg/mL glucose oxidase, 1% methylcellulose, 2% β-mercaptoethanol, final pH 7.4) 1:1. A mixture of 30 μL polymerizing actin was loaded into flow cells immediately and placed on a TIRF microscope (Leica Microsystems, Wetzlar, Germany). For nucleation assays, 8 different frames of actin nuclei were rapidly acquired and an average amount of actin nuclei was analyzed by using custom-written programs in MATLAB (The MathWorks, Natick, MA) R2017a. For polymerization assays, a time series of images of the actin polymerization process
were taken every 1 s for 5 min and the elongation rate was analyzed using ImageJ software (Version 1.49).

**Depolymerization assay**

For depolymerization assays, labeled G-actin was incubated 1:1 (v/v) with 1/10 volume of 10 x polymerization buffer (500 mM KCl, 10 mM MgCl₂, 10 mM EGTA, 1500 mM imidazole, final pH 7.1) and 1:8 with G-buffer at 25°C for 1 h to obtain labeled F-actin. 20 μL labeled F-actin (1:4 diluted with 1 x polymerization buffer) then were loaded into flow cells pre-blocked with 1% BSA and coated with 1 mg/mL α-actinin, washed with 50 μL of 1 x TIRF buffer containing 5 μM ChivoA and placed on a TIRF microscope immediately. Actin depolymerization was followed in time by collecting individual frames every 15 s for 90 min. Average length of actin filaments was quantified as parameter for depolymerization by using custom-written programs in MATLAB.

**Cell culture**

HUVECs (human umbilical vein endothelial cells) are primary cells purchased from Promocell, cultured with endothelial cell growth medium (Promocell, Heidelberg, Germany) under a constant humidity at 37°C and with 5% CO₂. The cells were used for functional assays at the 6th passage.

**Proliferation assay**

HUVECs (1.5×10³/well) were seeded in a 96-well plate, and treated with ChivoA or LatB, respectively, for 72 h. Cells were stained with 0.5% crystal violet solution (100 μL/well) for 10 min, then washed with H₂O and dried. 100 μL of 0.1 M Na-citrate solution were added to each
well, and incubated for 5 min on a bench rocker. Finally, the optical density in each well was measured at 550 nm with a plate reader (Sunrise, Tecan, Männedorf, Switzerland).

**Fluorescence staining**

HUVECs (25×10^3/well) were seeded in an ibidi μ slide (ibidi, Gräfelfing, Germany), and treated with ChivoA as indicated for 1 h. Then cells were fixed with 4% (v/v) paraformaldehyde and permeabilized with 0.2% Triton-X. Cells were stained with rhodamine phalloidin (0.5 units/mL) for F-actin and with Hoechst (1μg/mL) for nuclei. Cell images were taken using a LSM 510 META confocal microscope (Zeiss).

**Cell migration assay**

For scratch assays, HUVECs were grown to confluence, and the monolayer was wounded with a custom-made scratching device. Cells were treated with the indicated concentrations of compounds and allowed to migrate for 16 h. After migration, wells were washed with PBS (including Mg^{2+} and Ca^{2+}), stained with 0.5% Crystal Violet solution (100 μL/well) for 10 min, then washed with H_2O and dried. Images were taken by using an inverted microscope (Eclipse, Nikon). Cell migration was quantified as the percentage of cell covered area compared with total image area by using ImageJ.

For 2D-chemotaxis assays, HUVECs (5×10^3/well) were seeded into a μ-Slide Chemotaxis ibiTreat (ibidi). After 2 h, cells were treated with indicated concentrations of ChivoA and LatB, respectively, and a gradient of FCS between 0 and 10% (v/v) was applied. The cell migration process was imaged as time series (every 10 min for 20 h) by using an inverted Nikon microscope (Eclipse), equipped with an incubation chamber (ibidi). Humidity was kept at 80%,
temperature at 37ºC, and CO2 at 5%. Images were analyzed by using the Chemotaxis and Migration Tool (ibidi, Version 2.0). The parameters cells migration mean velocity, x-MFI (x-axis migration forward index) and directness were evaluated.

*G-actin binding assay*

The G-actin binding assay was performed by using the Actin-Toolkit G-Actin Binding (alpha skeletal muscle actin) from Hypermol. According to the manufacturer’s instructions, actin beads were incubated as indicated with our compounds at 25ºC for 30 min under agitation, then the respective actin binding proteins (ABPs) were added and incubated for 1 h. The mixture of beads, APBs and compounds were spun at 6,000 x g for 4 min, and 40 μL of the supernatant was taken up into SDS sample buffer. The pellet was washed, mixed with SDS sample buffer and boiled. The binding of ABPs to G-actin is highly specific, and thus, only ligands bound to G-actin will be co-precipitated under low centrifugal condition. Both supernatant and pellet were analyzed by SDS-PAGE and imaged on gel documentation system (Chemidoc, Biorad, Hercules, CA, USA). The amount of each ABP bound to the beads was quantified by using imageJ software (Version 1.49).

*Cross-linking assay*

G-actin (27 μM) was dissolved in crosslink G-buffer (3 mM triethanolamine-HCl, 0.2 mM CaCl2, 0.2 mM ATP, NaN3, pH 7.5) and incubated with either ChivoA or LatB as indicated at 4ºC for 30 min. Then thymosin β4 was applied and incubated at 4ºC for 45min at a weight ratio of 9:1, corresponding to 0.94 molecules of thymosin β4 per actin monomer. Aliquots of 10 μL were then mixed with 12.2μL of 5.4 mM EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) in 0.1 M MES (2-(N-morpholino)-ethanesulfonic acid), pH 6.5, and incubated for
2 h at 25°C. Equal aliquots were taken up into SDS sample buffer and analyzed by SDS-PAGE on a 10% gel. Cross-link product was quantified as a normalized gray intensity of cross-link product band compared with actin.

**Size-exclusion chromatography**

Lyophilised rabbit α-actin (Hypermol) was dissolved either in 20 mM MES, 150 mM NaCl, 0.2 mM ATP, 0.2 mM CaCl₂, pH 6.5 or 20 mM Tris-HCl, 150 mM NaCl, 0.2 mM ATP, 0.2 mM CaCl₂, pH 8. ChivoA was added to the actin (final concentrations: 120 µM actin, 350 µM ChivoA) and incubated on ice for 20 min. The solution was centrifuged (20 min, 4°C, 15,000 x g) and analysed by size exclusion chromatography (Äkta Pure, Superdex 200 10/300; GE Healthcare, Chicago, IL, USA).

**Transcriptome**

HUVECS at 80% confluency were treated with equipotent concentrations of ChivoA or LatB (20 nM and 250 nM, respectively) for 4 h. The concentrations were chosen in order to stay below levels causing visible alterations of cell morphology and overall actin structure. mRNA was cleaned up from cell lysates with Sera-Mag carboxylated magnetic beads (Thermo Fisher, Waltham, MA, USA) and reversely transcribed using a slightly modified SCRB-seq protocol (49). During reverse transcription, sample-specific barcodes and unique molecular identifiers were incorporated into first strand cDNA. Next, samples were pooled and excess primers digested by Exonuclease I (Thermo Fisher, Waltham, MA, USA). cDNA was preamplified using KAPA HiFi HotStart polymerase (KAPA Biosystems). Sequencing libraries were constructed from cDNA using the Nextera XT Kit (Illumina, San Diego, CA, USA). Resulting libraries were quantified and sequenced at 10 nM on a HiSeq1500 (Illumina, San Diego, CA, USA). To obtain
gene-wise expression values, raw sequencing data was processed using the zUMIs pipeline (49), using the Human genome build hg19 and Ensembl gene models (GRCh37.75). Transcriptome analysis was performed using the free statistical software R (v. 3.4.2). DESeq2 package (v.1.16.1) was used for normalization and differential expression (DE) analysis. DESeq2 models transcriptional count data using negative binomial distribution. Additional filtering was done using HTSFilter (v.1.16.0) to remove constant, lowly expressed genes. The final gene set consisted of 15,232 genes. DE testing was based on Wald test. Multiple testing was accounted for by applying a global false discovery rate (FDR) correction to all comparisons. All genes with FDR<0.1 were considered significant.

Quantification and statistical analysis

Quantitative data are expressed as mean ± SEM. Statistical analysis was performed with the software Graphpad Prism Version 7.02 (Graphpad Software, Inc., La Jolla, CA, USA). Statistical differences were evaluated by using Kruskal-Wallis test or one-way analysis of variance (ANOVA). P-values less than 0.05 were considered to be significant. For all tests, three independent replicates (n = 3) were used. Specific information on the statistical procedures used can be found in the respective figure legends.
**Supporting Information.** This file contains Supporting Figures S1 – S4 and Tables S1 and S2 (PDF).

**Corresponding Author**

* Sabine Schneider (structural biology): Department of Chemistry, Technical University Munich, 85748 Garching, Germany, email: sabine.schneider@mytum.de

* Stefan Zahler (cell biology and biochemistry): Department of Pharmacy, Ludwig-Maximilians-Universität Munich, 81377 Munich, email: Stefan.zahler@cup.uni-muenchen.de, Phone: ++4989218077196

**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. #These authors contributed equally.

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REFERENCES

1. Dominguez R (2004) Actin-binding proteins—a unifying hypothesis. Trends in biochemical sciences 29:572-578.
2. Caridi CP, et al. (2018) Nuclear F-actin and myosins drive relocalization of heterochromatic breaks. Nature 559(7712):54-60.
3. Schrank BR, et al. (2018) Nuclear ARP2/3 drives DNA break clustering for homology-directed repair. Nature 559(7712):61-66.
4. Yang X & Lin Y (2018) Functions of nuclear actin-binding proteins in human cancer. Oncol Lett 15(3):2743-2748.
5. Gandalovicova A, et al. (2017) Migrastatics-Anti-metastatic and Anti-invasion Drugs: Promises and Challenges. Trends Cancer 3(6):391-406.
6. Katagiri K & Matsuura S (1971) Antitumor activity of cytochalasin D. J Antibiot (Tokyo) 24(10):722-723.
7. Allingham JS, Klenchin VA, & Rayment I (2006) Actin-targeting natural products: structures, properties and mechanisms of action. Cell Mol Life Sci 63(18):2119-2134.
8. Klenchin VA, et al. (2003) Trisoxazole macrolide toxins mimic the binding of actin-capping proteins to actin. Nat Struct Biol 10(12):1058-1063.
9. Tanaka J, et al. (2003) Biomolecular mimicry in the actin cytoskeleton: mechanisms underlying the cytotoxicity of kabiramide C and related macrolides. Proc Natl Acad Sci USA 100(24):13851-13856.
10. Davidson AJ & Wood W (2016) Unravelling the Actin Cytoskeleton: A New Competitive Edge? Trends Cell Biol 26(8):569-576.
11. Irschik H, Jansen R, Gerth K, Hofle G, & Reichenbach H (1995) Chivosazol A, a new inhibitor of eukaryotic organisms isolated from myxobacteria. J Antibiot (Tokyo) 48(9):962-966.
12. Diestel R, et al. (2009) Chivosazoles A and F, cytostatic macrolides from myxobacteria, interfere with actin. ChemBioChem 10:2900-2903.
13. Janssen D, Albert D, Jansen R, Muller R, & Kalesse M (2007) Chivosazole A--elucidation of the absolute and relative configuration. Angew Chem Int Ed Engl 46(26):4898-4901.
14. Filipuzzi I, et al. (2017) Direct Interaction of Chivosazole F with Actin Elicits Cell Responses Similar to Latrunculin A but Distinct from Chondramide. ACS Chem Biol 12(9):2264-2269.

15. Allingham JS, Miles CO, & Rayment I (2007) A structural basis for regulation of actin polymerization by pectenotoxins. J Mol Biol 371(4):959-970.

16. Allingham JS, Tanaka J, Marriott G, & Rayment I (2004) Absolute stereochemistry of ulapualide A. Org Lett 6(4):597-599.

17. Allingham JS, Zampella A, D'Auria MV, & Rayment I (2005) Structures of microfilament destabilizing toxins bound to actin provide insight into toxin design and activity. Proc Natl Acad Sci U S A 102(41):14527-14532.

18. Blain JC, Mok YF, Kubanek J, & Allingham JS (2010) Two molecules of lobophorolide cooperate to stabilize an actin dimer using both their "ring" and "tail" region. Chem Biol 17(8):802-807.

19. Choe H, et al. (2002) The calcium activation of gelsolin: insights from the 3A structure of the G4-G6/actin complex. J Mol Biol 324(4):691-702.

20. Dominguez R & Holmes KC (2011) Actin structure and function. Annu Rev Biophys. 40:169--186.

21. Galkin VE, et al. (2011) Remodeling of actin filaments by ADF/cofilin proteins. Proc Natl Acad Sci U S A 108(51):20568-20572.

22. Hirata K, et al. (2006) Structure basis for antitumor effect of alyponine a. J Mol Biol 356(4):945-954.

23. Irobi E, et al. (2004) Structural basis of actin sequestration by thymosin-beta4: implications for WH2 proteins. EMBO J 23(18):3599-3608.

24. Pereira JH, et al. (2014) Structural and biochemical studies of actin in complex with synthetic macrolide tail analogues. ChemMedChem 9(10):2286-2293.

25. Hagelueken G, et al. (2009) The absolute configuration of rhizopodin and its inhibition of actin polymerization by dimerization. Angew Chem Int Ed Engl 48(3):595-598.

26. Klenchin VA, King R, Tanaka J, Marriott G, & Rayment I (2005) Structural basis of swinholide A binding to actin. Chem Biol 12(3):287-291.

27. Schutt CE, Myslik JC, Rozycki MD, Goonesekre NC, & Lindberg U (1993) The structure of crystalline profilin-beta-actin. Nature 365(6449):810-816.

28. Gurel PS, et al. (2017) Cryo-EM structures reveal specialization at the myosin VI-actin interface and a mechanism of force sensitivity. Elife 6.

29. Miyamoto K & Gurdon JB (2013) Transcriptional regulation and nuclear reprogramming: roles of nuclear actin and actin-binding proteins. Cell Mol Life Sci 70(18):3289-3302.

30. Carlier MF, et al. (1996) Tbeta 4 is not a simple G-actin sequestering protein and interacts with F-actin at high concentration. J Biol Chem 271(16):9231-9239.
31. Sawaya MR, et al. (2008) Multiple crystal structures of actin dimers and their implications for interactions in the actin filament. *Acta Crystallogr D Biol Crystallogr* 64(Pt 4):454-465.

32. Dawson JF, Sablin EP, Spudich JA, & Fletterick RJ (2003) Structure of an F-actin trimer disrupted by gelsolin and implications for the mechanism of severing. *J Biol Chem* 278(2):1229-1238.

33. Heisler DB, et al. (2015) ACTIN-DIRECTED TOXIN. ACD toxin-produced actin oligomers poison formin-controlled actin polymerization. *Science* 349(6247):535-539.

34. Brodmann T, Janssen D, & Kalesse M (2010) Total synthesis of chivosazole F. *J Am Chem Soc* 132(39):13610-13611.

35. Williams S, et al. (2017) An Expedient Total Synthesis of Chivosazole F: an Actin-Binding Antimitotic Macrolide from the Myxobacterium Sorangium Cellulosum. *Angew Chem Int Ed Engl* 56(2):645-649.

36. Sasse F, Steinmetz H, Hofle G, & Reichenbach H (1993) Rhizopodin, a new compound from Myxococcus stipitatus (myxobacteria) causes formation of rhizopodia-like structures in animal cell cultures. Production, isolation, physico-chemical and biological properties. *J Antibiot (Tokyo)* 46(5):741-748.

37. Kabsch W (2010) Integration, scaling, space-group assignment and post-refinement. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2):133-144.

38. Diederichs K & Karplus PA (2013) Better models by discarding data? *Acta Crystallogr D Biol Crystallogr* 69(Pt 7):1215-1222.

39. Evans P (2012) Biochemistry. Resolving some old problems in protein crystallography. *Science* 336(6084):986-987.

40. Karplus PA & Diederichs K (2012) Linking crystallographic model and data quality. *Science* 336(6084):1030-1033.

41. McCoy AJ, et al. (2007) Phaser crystallographic software. *J Appl Crystallogr* 40(Pt 4):658-674.

42. Adams PD, et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2):213-221.

43. Afonine PV, et al. (2012) Towards automated crystallographic structure refinement with phenix.refine. *Acta Crystallogr D Biol Crystallogr* 68(Pt 4):352-367.

44. Emsley P, Lohkamp B, Scott WG, & Cowtan K (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66(Pt 4):486-501.

45. Murshudov GN, et al. (2011) REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr D Biol Crystallogr* 67(Pt 4):355-367.

46. Stamper GF, Morollo AA, & Ringe D (1998) Reaction of alanine racemase with 1-aminoethylphosphonic acid forms a stable external aldimine. *Biochemistry* 37(29):10438-10445.
47. Krissinel E & Henrick K (2007) Inference of macromolecular assemblies from crystalline state. *J Mol Biol* 372(3):774-797.

48. Laskowski RA & Swindells MB (2011) LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. *J Chem Inf Model* 51(10):2778-2786.

49. Parekh S, Ziegenhain C, Vieth B, Enard W, & Hellmann I (2018) zUMIs - A fast and flexible pipeline to process RNA sequencing data with UMIs. *Gigascience* 7(6).
