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Identification of MAC1: A Small Molecule That Rescues Spindle Bipolarity in Monastrol-Treated Cells

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Abstract

The genetic integrity of each organism is intimately tied to the correct segregation of its genome during mitosis. Insights into the underlying mechanisms are fundamental for both basic research and the development of novel strategies to treat mitosis-relevant diseases such as cancer. Due to their fast mode of action, small molecules are invaluable tools to dissect mitosis. Yet, there is a great demand for novel antimitotic compounds. We performed a chemical genetic suppression screen to identify compounds that restore spindle bipolarity in cells treated with Monastrol, an inhibitor of the mitotic kinesin Eg5. We identified one compound—MAC1—that rescued spindle bipolarity in cells lacking Eg5 activity. Mechanistically, MAC1 induces the formation of additional microtubule nucleation centers, which allows kinesin Kif15-dependent bipolar spindle assembly in the absence of Eg5 activity. Thus, our chemical genetic suppression screen revealed novel unexpected insights into the mechanism of spindle assembly in mammalian cells.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.6b00203.

Supplemental figures and full experimental details (PDF)

Supporting Video 1a: BSC-1 cells stably expressing GFP- α -tubulin and histone H2B-mCherry were treated with 100 μ M Monastrol in order to induce monopolar spindle assembly. At time point 0, DMSO was added and spindle morphology in the presence of Monastrol was monitored. (AVI)

Supporting Video 1b: BSC-1 cells stably expressing GFP- α -tubulin and histone H2B-mCherry were treated with 100 μ M Monastrol in order to induce monopolar spindle assembly. At time point 0, 25 μ M MAC1 was added and spindle morphology in the presence of Monastrol was monitored. (AVI)

Supporting Video 2a: HeLa cells stably expressing EB3-GFP were incubated on ice for 1 h to disassemble all microtubules and during the last 10 min, DMSO was included in the medium. After changing to medium containing DMSO at 37°C, cells were immediately imaged to visualize microtubule growth. (AVI)

Supporting Video 2b: HeLa cells stably expressing EB3-GFP were incubated on ice for 1 h to disassemble all microtubules and during the last 10 min, 100 μ M MAC1 was included in the medium. After changing to medium containing 100 μ M MAC1 at 37°C, cells were immediately imaged to visualize microtubule growth. (AVI)

The authors declare no competing financial interest.

Graphical abstract

During mitosis, the previously duplicated genome is segregated such that each daughter cell receives an identical set of chromosomes.^{1,2} Errors in this process can lead to abnormal chromosome numbers, i.e. aneuploidy, a hallmark of most solid tumors.³ At the core of the cellular machinery mediating the process of chromosome segregation is the mitotic spindle, a bipolar structure composed of dynamic microtubule filaments.⁴ The shape and function of the mitotic spindle critically depends on the coordinated activities of kinesins and dyneins, molecular motor proteins that convert the energy released by ATP hydrolysis into mechanical work. Specifically, a balance between plus- and minus-end directed motors is required for bipolarity.^{5,6} Despite the fact that chromosome segregation has to occur with the highest possible fidelity, the process itself is highly dynamic and fast, taking less than an hour in mammalian cells.¹ The short duration and dynamicity of mitosis imposes a great challenge on the experimental tools applied to dissect the underlying processes. Due to their fast mode of action, small molecules have proven to be extremely powerful in the exploration of mitotic processes. Despite their great success, however, the number of appropriate compounds is still limiting, and therefore, there is a great demand for novel antimitotic compounds. Yet, the design of the optimal screen is decisive in identifying compounds that are suitable as tools to dissect biological processes.

In biology, genetic suppression screens—aimed to identify second site mutations reverting the original phenotype—are ideally suited to discover in a highly specific manner novel functional relationships between biological pathways. Therefore, to identify novel small molecules suitable to study mitosis, we decided to perform a chemical genetic suppression screen. In particular, we designed a screen to identify compounds that rescue spindle bipolarity in cells treated with the Eg5 inhibitor Monastrol.⁷ Eg5 is a motor protein whose activity is essential for the formation of bipolar spindles, and consequentially its inhibition results in spindle collapse and the formation of monopolar spindles.^{8–11} Based on the rationale of suppression screens,¹² we expected to identify compounds that restore spindle bipolarity by either activating pathways that synergize with Eg5 or inhibiting those that antagonize Eg5 (Figure 1a). Following the outlined scheme (Figure 1b), BSC-1 (African green monkey kidney) cells were synchronized in S-phase by a double thymidine treatment followed by a release into 100 μ M Monastrol. Eight hours after the release, compounds were transferred at a final concentration of 33 μ M. When most cells had entered mitosis (1.5 h after compound addition), cells were chemically fixed and chromatin structures visualized by Hoechst 33342 staining. Subsequently, images were taken using an automated screening

microscope. On the basis of the rationale that the arrangement of chromosomes in a 2D image appears disc-like in the case of a monopolar spindle but bar-like in bipolar spindles, we used the eccentricity—a measure of how much a given shape deviates from being circular—of the DNA signal as a surrogate for spindle morphology (Figure 1c). To validate the automated image analysis, each plate contained control wells with cells treated with only Monastrol (monopolar spindles, low eccentricity) or only the solvent control DMSO (bipolar spindles, high eccentricity). As a secondary readout, spindle shapes were visualized by α -tubulin immunofluorescence (IF) staining. After screening a 16 320 member compound library in duplicate, one compound—termed MAC1 (Monastrol Antagonizing Compound 1)—was identified that induced bar-like chromatin structures in Monastrol-treated cells (Figure 1d). α -Tubulin IF analysis confirmed that MAC1 rescued spindle bipolarity in Eg5 inhibited cells in a dose dependent manner (Figure 1e and f).

After confirming the chemical structure of MAC1 (Figure S1a–c), we further analyzed its mode of action. Defects in spindle function, as induced by compounds such as Monastrol, activate the spindle assembly checkpoint (SAC), a surveillance mechanism that monitors the attachment of chromosomes to the mitotic spindle and delays anaphase onset, i.e., chromosome segregation, as long as attachment defects persist.¹³ Therefore, quantifying the percentage of mitotic cells undergoing anaphase and the time mitosis takes are highly sensitive readouts for spindle function. First, we analyzed the fate of cells that entered mitosis in the presence of Monastrol and MAC1 or DMSO (Figure 2a). Live-cell analysis revealed that only $9 \pm 7\%$ of the cells that entered mitosis in the presence of Monastrol and DMSO performed anaphase, as evidenced by the formation of two daughter cells. On average, these cells spent 533 min in mitosis. In the presence of Monastrol and MAC1, a significantly higher percentage of mitotic cells were able to enter anaphase, and mitotic timing in these cells was reduced. Both effects occurred in a MAC1 dose-dependent manner and were evident in cancerous (A549, human lung cancer) as well as noncancerous (RPE1, human retinal pigment epithelial) cell lines (Figures 2a and S2a,b). Treatment with 100 μ M MAC1 alone had no significant effect on mitosis (data not shown). These data suggest that cells in the presence of MAC1 were able to assemble functional bipolar spindles despite Eg5 inhibition. Next, we analyzed if MAC1 can also rescue preformed monopolar spindles. To this end, MAC1 was added to Monastrol-treated cells that had already entered mitosis and therefore assembled monopolar spindles (Figure 2b). Indeed, the addition of MAC1 but not DMSO significantly increased the percentage of mitotic cells capable of completing mitosis and reduced mitotic duration (Figure 2b). Collectively, these data suggest that MAC1 can not only prevent the formation of monopolar spindles but also restore spindle bipolarity in Eg5- inhibited cells mitotically trapped with monopolar spindles. Next, we sought to exclude that MAC1 rescues spindle bipolarity by interfering with Monastrol, i.e. by suppressing its inhibitory activity toward Eg5 or inducing its efflux from cells. To test the former, we performed *in vitro* assays to quantify the ATPase activity of recombinant full-length Eg5 in the presence of Monastrol and increasing concentrations of MAC1 (Figure S2c). The microtubule-stimulated ATPase activity of Histagged Eg5 in the presence of DMSO was set to 100%. As expected, 32 μ M Monastrol significantly inhibited the ATPase activity of Eg5, and MAC1 even at concentrations as high as 200 μ M did not interfere with Monastrol's inhibitory effect (Figure S2d). To exclude that MAC1 interferes with the inhibitory activity

of Monastrol in cells, we analyzed if MAC1 is capable of rescuing spindle bipolarity in cells that lack Eg5 activity due to RNA interference (RNAi) mediated depletion of Eg5. While $100 \pm 0\%$ of control-depleted RNAi cells that entered mitosis progressed through anaphase, only a minor fraction of DMSO-treated Eg5-RNAi cells were able to do so (Figures 2c and S2e and data not shown). Likewise, mitotic timing was significantly increased in these cells. Importantly, treatment with MAC1 rescued the mitotic defects of Eg5-RNAi cells, and this effect was dose-dependent. In summary, these data indicate that MAC1 does not interfere with the action of Monastrol but rather acts on the process of spindle bipolarization in cells lacking Eg5 activity.

To gain insight into the mechanism of MAC1, we first analyzed in detail the conversion of preformed monopolar spindles in Monastrol-treated cells expressing GFP- α -tubulin and histone H2B-mCherry, as a marker for chromatin (Figure 2d, Supporting Video 1a,b). Upon the addition of DMSO (time point 0), spindles remained monopolar throughout the experiment with chromosomes forming a radial array. Upon the addition of MAC1, monopolar spindles started to elongate followed by the movement of chromosomes toward the spindle equator, their alignment at the metaphase plate, and finally the segregation of chromosomes. Intriguingly, closer inspection revealed that spindle bipolarization in MAC1-treated cells was preceded by the formation of foci (inset, white arrow heads), which were reminiscent of microtubule organizing centers (MTOC) in that MTs seemed to emanate from these foci. These centers were hardly—if at all—detectable in DMSO-treated cells. Under normal cellular conditions, MT nucleation is an unfavorable reaction and therefore depends on γ -tubulin, which as part of the γ -tubulin ring complex (γ -TuRC) acts as a template that facilitates the nucleation of MTs.^{14–17} Centrosomes, which like the genome duplicate during S-phase of the cell cycle, serve as prime MTOCs by recruiting γ -TuRCs and organizing the MTs nucleated from there into a cell-cycle specific structure: the characteristic radial array of MTs in interphase and the bipolar spindle in the presence of two centrosomes in mitosis.^{18,19} To investigate if the foci detected upon MAC1 treatment were *bona fide* centrosomes, we stained for centrin, a centriolar marker protein characteristic for centrosomes.²⁰ As expected, mitotic cells treated with only Monastrol contained two centrosomes recognizable by two γ -tubulin foci, each of which contained two centrioles as evident by centrin staining (Figure S3a). Upon treatment with MAC1, multiple γ -tubulin foci were detectable. However, only the two most prominent γ -tubulin foci were positive for centrin, while the other ones were devoid of a detectable centrin signal, indicating that MAC1 treated cells contained normal numbers of centrosomes. Thus, these data suggest that MAC1 induces the formation of acentrosomal, γ -tubulin positive MT nucleation centers.

Next, we analyzed how MAC1 affects microtubule nucleation. We therefore performed MT regrowth assays to analyze in more detail the mechanism of MAC1 induced MT nucleation centers. In brief, cells were incubated on ice to depolymerize all existing MTs, treated with DMSO or MAC1, and after rewarming to 37 °C, MT regrowth was analyzed by IF microscopy (Figure 3a). Since MTs are more dynamic in mitosis and therefore more difficult to analyze, we focused our analyses on interphase cells. Consistent with the function of centrosomes as prime MT nucleation centers, most MTs in DMSO-treated cells were radiated from a single center in the cytoplasm (Figure 3a). In strong contrast, in MAC1 treated cells, MTs nucleated not only from the centrosome but also throughout the

cytoplasm, forming a meshwork of short filaments. To quantify this effect, we determined the intensity of the MT signal per cell by IF microscopy. Analyses of cells fixed 2 min after rewarming confirmed that MAC1-treated cells contained significantly more polymerized MTs than DMSO control cells (Figure 3b). To corroborate this result, we performed live-cell analyses of cells stably expressing GFP-tagged EB3, a MT associated protein that decorates the tips of growing MTs.²¹ Using EB3-GFP as a marker for growing MTs,²² we confirmed that MT growth occurred almost exclusively from centrosomes in DMSO treated cells, whereas in MAC1 treated cells MTs were nucleated throughout the cytoplasm (Supporting Video 2a,b).

Next, we sought to confirm that MAC1 stimulated MT nucleation in a γ -tubulin dependent manner. To this end, we repeated the MT regrowth assay in cells depleted of γ -tubulin. While MAC1 induced MT nucleation throughout the cytoplasm in control-depleted cells, depletion of γ -tubulin by RNAi prevented MAC1 induced acentrosomal MT nucleation (Figure S3b). Note that γ -tubulin is not completely depleted (Figure S3c), explaining the residual MT nucleation capacity of centrosomes. These data indicate that MAC1 induces acentrosomal MT nucleation in a γ -tubulin dependent manner.

Based on these data, we speculated that acentrosomal MT nucleation accounts for MAC1's ability to rescue spindle bipolarity. If true, induction of acentrosomal MT nucleation by any means should rescue bipolar spindle formation in Eg5-inhibited cells. To test this hypothesis, we generated stable cell lines expressing mCherry-tagged γ -TuNA (γ -TuRC-mediated nucleation activator), a fragment of the centrosomal protein CDK5RAP2, which binds to the γ -TuRC and stimulates its MT nucleation activity.²³ Like MAC1 and consistent with previous reports, expression of γ -TuNA induced ectopic MT nucleation in the MT regrowth assay (Figure 3c). Importantly, as evidenced by live-cell imaging, expression of γ -TuNA rescued the mitotic defect of Monastrol-treated cells (Figure 3d). These data strongly support the idea that MAC1 rescues spindle bipolarity by inducing acentrosomal MT nucleation, resulting in the formation of additional MT nucleation centers. This idea raises the question of how the formation of additional nucleation centers facilitates spindle bipolarization in cells lacking Eg5-activity. As shown previously, kinesin Kif15 can take over the function of Eg5 in separating spindle poles.^{24–27} We therefore speculated that the formation of ectopic MT nucleation centers enables Kif15 to establish spindle bipolarity. To test this idea, we analyzed if RNAi depletion of Kif15 affects the ability of MAC1 to restore spindle function in cells lacking Eg5 activity. Live-cell analyses revealed that cells depleted of Kif15 and treated with DMSO efficiently entered anaphase (Figure 4a), confirming previous studies reporting that Kif15 function is dispensable in the presence of Eg5 activity. Intriguingly, Kif15 depletion (Figure 4b) significantly reduced the ability of MAC1 to restore spindle function in Monastrol-treated cells. Live-cell analyses of GFP- α -tubulin and H2BmCherry expressing cells confirmed that spindles remained monopolar in Kif15-RNAi cells treated with Monastrol and MAC1 (Figure S4b,c). Notably, consistent with the idea that the formation of ectopic nucleation centers precedes the process of Kif15 mediated spindle pole separation, depletion of Kif15 did not affect MAC1's ability to induce MT nucleation in the MT regrowth assay (Figure S4a).

In summary, our chemical genetic suppression screen identifies MAC1 as a suppressor of spindle monopolarity in cells lacking Eg5 activity. In wildtype cells, Eg5 separates the two spindle poles and thereby establishes spindle bipolarity. Inactivation of Eg5 either by small molecule treatment or RNAi mediated depletion of Eg5 results in monopolar spindles. Based on our results, we postulate that MAC1 rescues bipolarity by inducing ectopic MT nucleation, thereby creating an auxiliary MT network (Figure 4c). This network of overlapping MTs enables Kif15 to produce force and separate the two centrosomes. Concomitantly, ectopic nucleation centers coalesce into the two separating spindle poles, resulting ultimately in the establishment of spindle bipolarity.

Intriguingly, previously performed suppression screens based on RNAi mediated protein depletion missed that the formation of acentrosomal MT nucleation centers is capable of rescuing spindle bipolarity in cells lacking Eg5 activity.¹² It is conceivable that the low temporal resolution of RNAi-based screens—it typically takes days to deplete a protein—accounts for the failure to detect this process. In contrast, MAC1—like most small molecules—acts on a fast time scale, resulting in acute inhibition of the target protein. This temporal resolution can be critical if a given biological process has to be inactivated at a distinct time point. Thus, while the molecular target of MAC1 has yet to be identified, MAC1 proves the power of chemical genetic suppression screens to identify unexpected functional relationships between biological processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Identification of MAC1 (Monastrol Antagonizing Compound 1). (a) Cartoon of spindle morphologies in wildtype and Eg5 inhibited cells. Compound mediated activation of Eg5 synergy or inhibition of Eg5 antagonism is expected to induce spindle bipolarity in Eg5 inhibited cells. (b) Experimental outline of the chemical genetics suppression screen. (c) Image depicting the rationale of using the eccentricity value of the DNA signal as a surrogate for spindle morphology. (d) Chemical structure of MAC1. (e) Immunofluorescence images of cells treated with Monastrol (MA) and DMSO or MAC1. DNA and α-tubulin are shown in blue and green, respectively. Scale bar: 10 μm. (f) Quantitation of the percentage of bipolar spindles in cells treated as indicated (mean ± SD). For each condition, about 900 cells from three independent experiments were quantified. For details, see the Experimental Section.

Figure 2.

Characterization of MAC1 induced spindle rescue. (a) Upper panel: scheme of the experiment depicting that cells were treated with Monastrol and DMSO or MAC1 prior to entry into mitosis. Middle and lower panels: quantitation of live-cell analyses of cells treated as indicated. For each condition, 150 cells from three independent experiments were quantified. Middle panel represents the percentage of mitotic cells undergoing anaphase (mean $\pm$ SD); lower panel, the time cells spent in mitosis (each dot represents one cell, and the mean is indicated by a horizontal bar). (b) Upper panel: scheme of the experiment

depicting that Monastrol-treated cells that already had assembled monopolar spindles were treated with DMSO or MAC1. Middle and lower panels show the same quantitation as in a. (c) Upper panel: scheme of the experiment depicting that cells depleted of Eg5 protein by RNAi were treated with DMSO or MAC1 prior to mitotic entry. Middle and lower panels show the same quantitation as in a. (d) Stills of live-cell movies of Monastrol-treated cells stably expressing GFP- α -tubulin and histone H2B-mCherry. At time point 0 min, DMSO or MAC1 was added to Monastrol-treated cells that had already assembled monopolar spindles and the fate of spindles monitored over time. Inset shows magnified image with white arrowheads pointing at additional MT nucleation centers. See also Supporting Video 1a,b. Scale bar: 10 μ m.

Figure 3.

Mechanism of MAC1 induced spindle rescue. (a) Upper panel: experimental outline of MT regrowth assay. Lower panel: immunofluorescence (IF) images of interphase cells treated with DMSO or MAC1 and processed for IF analyses at indicated time points after rewarming to 37 °C. α -Tubulin and DNA are shown in green and blue, respectively. Insets show magnification of regrown cytoplasmic microtubules. Scale bar: 10 μ m (b) Quantitation of the microtubule signal intensity in cells treated as outlined in a and rewarmed to 37 °C for 2 min. Shown are means $\pm$ SD of six independent experiments (for details, see Experimental

Section). (c) IF images of wildtype (wt) cells and cells expressing mCherry- γ -TuNA after cold-induced MT depolymerization and rewarming to 37 °C for 30 s. α -Tubulin and DNA are shown in green and blue, respectively, and expression of mCherry-tagged proteins in red. Insets show magnification of cytoplasmic MTs. (d) Upper panel: experimental outline. Lower panel: live-cell quantitation of mitotic fate of parental cells (wt) or cells stably expressing mCherry- γ -TuNA. Shown is the percentage of cells that underwent anaphase in the presence of Monastrol. Quantified are 150 cells per condition from three independent experiments (means $\pm$ SD).

Figure 4.

Model of spindle rescue. (a) Left panel: experimental outline depicting that Kif15 or control (ctrl) depleted cells were treated as indicated prior to entry into mitosis. Right panel: Live-cell quantitation of mitotic cells undergoing anaphase. Cells were treated as indicated. Quantified are 150 cells per condition from three independent experiments (means $\pm$ SD). (b) Kif15 Western blot of ctrl or Kif15 depleted cells. The α -tubulin signal serves as loading

control. (c) Model of spindle bipolarization in wildtype cells (upper pathway) or MAC1 induced spindle bipolarization in cells lacking Eg5 activity (lower pathway).

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