MLN8054, a Small-Molecule Inhibitor of Aurora A, Causes Spindle Pole and Chromosome Congression Defects Leading to Aneuploidy††

Kara Hoar,1 Arijit Chakravarty,2 Claudia Rabino,1 Deborah Wysong,1 Douglas Bowman,1 Natalie Roy,1 and Jeffrey A. Ecsedy1∗

Departments of Molecular and Cellular Oncology1 and Cancer Pharmacology,2 Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts 02139

Received 19 December 2006/Returned for modification 12 February 2007/Accepted 3 April 2007

Aurora A kinase plays an essential role in the proper assembly and function of the mitotic spindle, as its perturbation causes defects in centrosome separation, spindle pole organization, and chromosome segregation. Moreover, Aurora A disruption leads to cell death via a mechanism that involves aneuploidy generation. However, the link between the immediate functional consequences of Aurora A inhibition and the development of aneuploidy is not clearly defined. In this study, we delineate the sequence of events that lead to aneuploidy following Aurora A inhibition using MLN8054, a selective Aurora A small-molecule inhibitor. Human tumor cells treated with MLN8054 show a high incidence of abnormal mitotic spindles, often with unseparated centrosomes. Although these spindle defects result in mitotic delays, cells ultimately divide at a frequency near that of untreated cells. We show that many of the spindles in the dividing cells are bipolar, although they lack centrosomes at one or more spindle poles. MLN8054-treated cells frequently show alignment defects during metaphase, lagging chromosomes in anaphase, and chromatin bridges during telophase. Consistent with the chromosome segregation defects, cells treated with MLN8054 develop aneuploidy over time. Taken together, these results suggest that Aurora A inhibition kills tumor cells through the development of deleterious aneuploidy.

The mitotic spindle is a dynamic protein machine dedicated to the accurate segregation of genetic material during cell division. The spindle is comprised of microtubules, cylindrical heteropolymers of tubulin subunits with a dynamically unstable plus end and a relatively stable minus end. Microtubule minus ends are focused at the spindle poles by an ensemble of motor and structural proteins working in concert (for reviews, see references 13 and 48). Centrosomes, when present, act as the dominant sites for microtubule nucleation, but are dispensable for microtubule minus-end focusing in many systems (reviewed in reference 48). During prometaphase, the plus ends of microtubules grow and shrink rapidly, eventually attaching themselves to condensed chromosomes. Microtubule-chromosome interactions occur both along the chromosome arms and at the kinetochore, a specialized protein assembly that serves at once as a point of attachment and as a sensor of attachment integrity. Kinetochore capture by spindle microtubules drives chromosome congression towards the metaphase plate. After the last kinetochore attaches to the spindle, the linkage between sister chromatids is dissolved, and the subsequent rapid poleward movement of chromatids during anaphase ultimately gives rise to two identical daughter cells.

The spatiotemporal choreography of the mitotic spindle assembly process is regulated by a number of serine/threonine protein kinases, which phosphorylate the structural and motor proteins required for spindle assembly, as well as those required for the coordinated onset of anaphase and proper completion of cell division. Among these are the Aurora-related kinases, which in mammalian cells include Aurora A and Aurora B. The role of the Aurora kinases in mitosis has been extensively studied in recent years (reviewed in references 17, 37, and 39). Although the products of these paralogous genes maintain a high degree of structural similarity in their kinase domains, Aurora A and Aurora B have distinct functions during mitosis, which are foreshadowed by their distinct localization patterns.

Aurora B is dynamically associated with the centromeres of preanaphase cells, the spindle midzone of anaphase cells, and the midbody of telophase cells (10, 40; reviewed in references 8 and 37). In keeping with its localization, Aurora B plays critical roles in the establishment and maintenance of spindle bipolarity, the spindle assembly checkpoint, and cytokinesis. Disruption of Aurora B function, by small molecules, RNA interference (RNAi), or dominant-negative mutants, causes defects in kinetochore microtubule attachments in prometaphase, chromosome movement during anaphase, and cleavage furrow formation during telophase (1, 11, 15, 26, 41, 51). Inhibition of Aurora B activity abrogates the spindle assembly checkpoint, leading to exit from mitosis with no evidence of either anaphase or cytokinesis and without a mitotic arrest (11, 24).

Aurora A localizes to centrosomes and the proximal mitotic spindle during mitosis (10; reviewed in references 8 and 37). Previous reports have shown Aurora A to be involved in a diverse set of processes during mitosis: centrosome maturation, centrosome separation, the G2/M transition, the formation of spindle poles and spindles, chromosome alignment and
separation, the spindle assembly checkpoint, and cytokinesis (reviewed in references 7, 8, 18, 22, and 42).

One consequence of Aurora A inhibition is the failure of cells to properly align and segregate their chromosomes. The results of studies in a number of different systems using genetic mutants, RNAi, and antibody microinjection show that Aurora A inhibition leads to defects in chromosome congression during prometaphase (21, 28, 36). These defects are usually concomitant with a mitotic delay that is thought to occur via the spindle assembly checkpoint. Some cells lacking functional Aurora A are able to divide in the presence of unaligned chromosomes (36), possibly due to their inability to maintain a stable spindle assembly checkpoint arrest. Further, cells lacking functional Aurora A frequently develop segregation defects in anaphase and chromatin bridges at telophase, resulting in abnormal mitotic spindles formed around abnormally large centrosomes (21). It is interesting to note that at least some cells in these mutants are capable of progressing to metaphase with bipolar spindles, although they subsequently develop lagging chromosomes in anaphase and fail to complete telophase.

Thus, although various functional consequences of Aurora A inhibition have been well documented for a variety of cell types, a mechanistic picture of the sequence of events connecting Aurora A inhibition to cell death is lacking. To gain a clearer understanding of the mechanism of cell death in the absence of Aurora A function, we used a novel small-molecule inhibitor of Aurora A, MLN8054 (35). MLN8054 inhibits the Aurora A enzyme selectively relative to over 200 other kinases screened. MLN8054 inhibits Aurora A autophosphorylation on Thr288 in cells, results in G2/M accumulation, and induces abnormal mitotic spindles, phenotypes consistent with known Aurora A inhibition. Moreover, MLN8054 is more than 150-fold more selective for Aurora A than for the family member Aurora B in cultured cells. The use of a small-molecule inhibitor offers significant advantages over both small interfering RNA (siRNA) inhibition and antibody microinjection, since Aurora A inhibition by MLN8054 occurs rapidly and is reversible. Specifically, we sought to characterize and quantify the spindle pole and centrosomal defects over time and their impact on cell division in response to Aurora A inhibition via MLN8054. We further sought to trace the fates of cells that had undergone division subsequent to MLN8054 treatment. The results presented here are consistent with a model in which Aurora A inhibition leads to eventual cell death via deleterious aneuploidy.

MATERIALS AND METHODS

Cell culture and compound treatment. HCT-116, H460, and DLD1 human tumor cells were obtained from the American Type Culture Collection and maintained according to the distributor’s recommendations. MLN8054 was diluted in distilled water and added to the cell culture medium at a final concentration of 0.25 μM. Bortezomib (Velcade; Millennium Pharmaceuticals, Inc.) was diluted in dimethyl sulfoxide (DMSO) and added to the cell culture medium at a final concentration of 100 nM.

Immunofluorescence staining. HCT-116 cells grown on glass coverslips and treated as described above were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) and then permeabilized with 0.5% Triton X-100 (Sigma) in phosphate-buffered saline (PBS). Blockage reagent (Roche) was added to cells for 30 min, followed by a 1-h incubation with the primary antibody in blocking reagent. The cells were washed in PBS and in PBS containing 0.05% Tween, followed by the addition of the secondary antibody in blocking reagent for 1 h. The cells were then washed with PBS and incubated with Hoechst 33342 in PBS (1:10,000; Molecular Probes) for 5 min. The cells were washed twice in PBS and mounted on glass slides with Fluoromount G (Electron Microscopy Sciences). The primary antibodies used in these studies included anti-α-tubulin mouse antibody (1:1,000; Sigma), anti-α-tubulin rabbit antibody (1:1,000; Abcam), anti-IAK1 (LI1 and Aurora-related kinase 1) mouse antibody (1:250; BD Transduction Laboratories), anti-pericentriolar plasma membrane A (1:500; Abcam), anti-nuclear and mitotic apparatus protein antibody (anti-NuMA) mouse antibody (1:250; Calbiochem), anti-γ-tubulin rabbit antibody (1:250; Sigma), and nuclear ANA (antinuclear clear antigen)-centromere autoantibody (CREST) (1:1,000; Cortico Biochem) to stain kinetochores. The secondary antibodies used in these studies included Alexa 488-conjugated goat anti-mouse immunoglobulin G (IgG) (1:250; Molecular Probes), Alexa 488-conjugated goat anti-rabbit IgG (1:250; Molecular Probes), Alexa 594-conjugated goat anti-mouse IgG (1:250; Molecular Probes) and Alexa 594-conjugated goat anti-rabbit IgG (1:250; Molecular Probes). Immunofluorescently labeled cells were visualized with 63× or 100× objectives and an LSM 510 Pa laser-scanning confocal microscope (Zeiss). The images were processed using MetaMorph software.

Abnormal mitotic spindle, centrosome, and spindle pole quantification. The percentage of abnormal mitotic spindles was determined by evaluating mitotic spindle architecture from the immunofluorescently stained images. Abnormal spindles were defined as those that did not display canonical bipolar spindle formation, as defined by the existence of a clearly visible metaphase plate straddled by undischarged radial arrays of microtubules emanating from opposite poles. Centrosomes and spindle poles were quantified by determining the number of distinct pericentrin- and NuMA-immunopositive spots, respectively, per mitotic cell.

Aneuploidy quantification. HCT-116 cells were treated with DMSO or 0.25 μM MLN8054 for 24, 48, or 72 h. Aphidicholin (5 nM) was added for the final 16 h to arrest cells in the G1 cell cycle phase. Kinetochores, α-tubulin, and DNA were immunofluorescently labeled as described above and imaged with an E800 microscope (Nikon Instruments) equipped with an automated XYZ stage (Prior Scientific), a filter wheel (Sutter Instruments), and a Cool Snap HQ camera (Roper Scientific) controlled by MetaMorph software (Molecular Devices). Z sections were acquired at 0.1-μm intervals with a 60× objective. Z-stack images were processed using MetaMorph software and compressed to single best-fit images. Grossly abnormal interphase nuclei were characterized as those containing more than one distinct nucleus per cell or were dramatically misshapen. The number of kinetochores per cell was quantitated by automated image processing using MetaMorph software.

Video microscopy. HCT-116 cells constitutively expressing enhanced green fluorescent protein (EGFP)-conjugated α-tubulin and mRFP-conjugated histone H2B were treated with DMSO or 0.25 μM MLN8054. Upon compound treatment, cells were added to a humidified chamber at 37°C with 5% CO2. Treatment was performed using MetaMorph software and compressed to single best-fit images. Grossly abnormal interphase nuclei were characterized as those containing more than one distinct nucleus per cell or were dramatically misshapen. The number of kinetochores per cell was quantitated by automated image processing using MetaMorph software.

RNAi. Suspended HCT-116 tumor cells (2 × 106) were transfected with 50 nM of GL2 (sense, 5'-CGUACCGGGAUAUCUUGCA-3') or Aurora A (sense, 5'-AUGCCCUUGUCUACUGUCA-3') siRNAs (Dharmacon) using 2 μl Lipofectamine 2000 reagent (Invitrogen) and 98 μl Opti-mem I (Invitrogen) on 6-well BioCoat cell culture plates (BD Biosciences) containing poly-lysine-coated coverslips. The cells were harvested 5, 24, and 48 h after transfection and processed for immunofluorescence and Western blot analysis.

Western blots. The cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl...
FIG. 1. Aurora A inhibition or ablation results in the formation of abnormal mitotic spindles. Abnormal spindles were defined as those that did not display canonical bipolar spindle formation, as defined by the existence of a clearly visible metaphase plate straddled by undisturbed radial arrays of microtubules emanating from opposite poles. (A and B) HCT-116 cells were treated with DMSO or 0.25 μM MLN8054 for 5, 24, and 48 h. A final concentration of 100 nM bortezomib was added for the final 2 h to arrest mitotic cells at the metaphase-to-anaphase transition. (A) Representative immunofluorescent images of mitotic spindles obtained from cells stained with anti-α-tubulin rabbit antibody and Hoechst stain (DNA). Four example MLN8054-treated mitotic cells are shown. The scale bars represent 10 μm. (B) The percentages of abnormal spindles were quantified 5, 24, and 48 h after treatment with DMSO or MLN8054. The P values demonstrate a statistical difference between control- and Aurora MLN8054-treated cells in the incidence of abnormal spindles at all time points. (C and D) HCT-116 cells were transfected with control (luciferase [Luc]) or Aurora A siRNAs for 5, 24, or 48 h. A 100 nM concentration of bortezomib was added for the final 2 h to arrest mitotic cells at the metaphase-to-anaphase transition. (C) Western blot probed for Aurora A and β-actin, which served as a protein-loading control. (D) The percentages of abnormal spindles were quantified 5, 24, and 48 h after siRNA transfection. The P values demonstrate a statistical difference between control- and Aurora A siRNA-transfected cells in the incidence of abnormal spindles at all time points.
FIG. 2. Aurora A inhibition delays mitotic progression, but cells ultimately divide at a high frequency. HCT-116 cells constitutively expressing EGFP–α-tubulin and mmiRed-histone H2B were treated with DMSO (A) or 0.25 μM MLN8054 (B). Images were captured every 5 min using a
IgG conjugated to horseradish peroxidase (1:10,000; Southern Biotechnologies). Anti-IAK1/Aurora A kinase mouse antibody (1:250) and anti-sham Biosciences). The primary antibodies used for Western blotting included 

Statistical analysis. The statistical significance (P value) for various experiments was assessed either by using a one-sided t test.

RESULTS

MLN8054 and Aurora A RNAi lead to defects in mitotic spindle formation. MLN8054 is an ATP-competitive selective Aurora A inhibitor that completely inhibits Aurora A in cells at 0.25 μM without affecting Aurora B activity (35). The effect of Aurora A inhibition via MLN8054 on spindle organization and chromosome alignment was examined by using immuno-fluorescence staining (Fig. 1). HCT-116 cells were exposed to 0.25 μM MLN8054 for 5, 24, or 48 h, and the proteasome inhibitor bortezomib was added 2 h before fixation to trap mitotic cells immediately prior to anaphase onset so that pro-metaphase cells, with their accompanying incomplete spindle formation and chromosome alignment, would not be construed as being abnormal. MLN8054-treated cells showed a variety of spindle organization defects after 24 h, not as monopolar as normal spindles were observed at 5 h, consistent with the observations that Aurora A participates in centrosome maturation and separation. However, these cells frequently presented with two centrosomes and two spindle poles per cell (Fig. 3A and B). 

Formation of abnormal mitotic spindles was also quantified in cells depleted of Aurora A by RNAi. Aurora A depletion was partial 5 h after siRNA transfection and complete 24 and 48 h after siRNA transfection (Fig. 1C). The percentages of abnormal spindles formed in HCT-116 cells upon transfection with Aurora A siRNA for 24 and 48 h were similar to those observed with MLN8054 treatment (Fig. 1D). Fewer abnormal spindles were observed at 5 h, consistent with the partial Aurora A depletion at this time point. These results demonstrate that the spindle defects observed in MLN8054-treated cells are similar to those obtained upon RNAi-mediated Aurora A depletion.

Cells treated with MLN8054 divide despite spindle abnormalities. To visualize the cell cycle progression of cells treated with 0.25 μM MLN8054, we used HCT-116 cells constitutively expressing EGFP-α-tubulin and mnRed-histone H2B. Cells treated with DMSO or 0.25 μM MLN8054 were imaged every five minutes for over 24 h using time-lapse microscopy (see Videos S1 and S2 in the supplemental material). Examples of control- and MLN8054-treated cells progressing through mitosis are shown in Fig. 2A and B, respectively. All of the tracked control-treated cells underwent an apparently normal division (Fig. 2A). However, some cells treated with MLN8054 undergo an abnormal division, displaying disorganized chromosomes at division (Fig. 2B; 3:05 h, cell B1) and micronuclei formation (Fig. 2B; 25:25 h, cell B2a).

MLN8054 treatment prolonged mitosis, increasing the average time from prophase to telophase from 67 to 131 min for the first mitotic division following treatment (Fig. 2C). A similar delay was observed at the second mitotic division (Fig. 2E). There was also an increase in overall cell cycle time, from 23 to 31 h, although this difference did not achieve statistical significance. A large percentage (65.5%) of MLN8054-treated cells completed cytokinesis; this was slightly lower than the percentage of cells in the control sample that divided in the same time frame (75.5%). From the observation that approximately 82% of the spindles in an MLN8054-treated sample at 24 h are abnormal (Fig. 1B), we may infer that a minimum of 58% of cells presenting with abnormal spindles underwent division within this time frame. This result was further corroborated using two other cell types. DLD1 and H460 cells also divide in the presence of MLN8054, although some cells appear to die during or shortly after division (see Fig. S1 and S2 in the supplemental material). Collectively, these results suggest that cells lacking functional Aurora A are capable of dividing despite the presence of spindle organization defects.

Cells treated with MLN8054 are able to establish bipolar spindles in the absence of centrosome separation. Aurora A plays an important role in centrosome maturation and separation (16, 21), and many of the MLN8054-treated cells observed by us at early time points have monopolar spindles. Despite this, MLN8054-treated cells were found to undergo division at a rate near that of control-treated cells. To understand this apparent paradox, we examined the centrosomes and spindle poles in HCT-116 cells treated with 0.25 μM MLN8054 for 5 h and bortezomib for the final 2 h. Cells were fixed and stained for centrosomes by using pericentrin and γ-tubulin and for spindle poles by using NuMA (Fig. 3). Control-treated cells at the metaphase-to-anaphase transition presented primarily with two centrosomes and two spindle poles per cell (Fig. 3A and B and 4). The MLN8054-treated cells frequently presented with only one centrosome per mitotic cell (Fig. 3A and B), consistent with the observations that Aurora A participates in centrosome maturation and separation. However, these cells frequently formed acentrosomal spindle poles (NuMA positive), forming two or more spindle poles per cell, where centrosomal markers were detected at only one of the poles (Fig. 3A and B and 4).

MLN8054 treatment prolonged mitosis, increasing the average time from prophase to telophase from 67 to 131 min for the first mitotic division following treatment (Fig. 2C). A similar delay was observed at the second mitotic division (Fig. 2E). There was also an increase in overall cell cycle time, from 23 to 31 h, although this difference did not achieve statistical significance. A large percentage (65.5%) of MLN8054-treated cells completed cytokinesis; this was slightly lower than the percentage of cells in the control sample that divided in the same time frame (75.5%). From the observation that approximately 82% of the spindles in HCT-116 cells treated with 0.25 μM MLN8054 at 5 h and bortezomib for the final 2 h. Cells were fixed and stained for centrosomes by using pericentrin and γ-tubulin and for spindle poles by using NuMA (Fig. 3). Control-treated cells at the metaphase-to-anaphase transition presented primarily with two centrosomes and two spindle poles per cell (Fig. 3A and B and 4). The MLN8054-treated cells frequently presented with only one centrosome per mitotic cell (Fig. 3A and B), consistent with the observations that Aurora A participates in centrosome maturation and separation. However, these cells frequently formed acentrosomal spindle poles (NuMA positive), forming two or more spindle poles per cell, where centrosomal markers were detected at only one of the poles (Fig. 3A and B and 4).
FIG. 3. Aurora A inhibition results in centrosome and spindle pole defects. HCT-116 cells were treated with DMSO (control) or 0.25 μM MLN8054 for 5 h. Two example MLN8054-treated cells are shown in each panel. A 100 nM concentration of bortezomib was added for the final 2 h to arrest mitotic cells at the metaphase-to-anaphase transition. (A) Representative immunofluorescent images of centrosomes and spindle poles from cells stained with anti-pericentrin rabbit antibody, anti-NuMA mouse antibody, and Hoechst stain (DNA). (B) Representative immunofluorescent images of centrosomes and spindle poles from cells stained with anti-γ-tubulin rabbit antibody, anti-NuMA mouse antibody, and Hoechst stain (DNA). (C) Representative immunofluorescent images of mitotic spindles and spindle poles from cells stained with anti-α-tubulin rabbit antibody, anti-NuMA mouse antibody, and Hoechst stain (DNA). (D) Representative immunofluorescent images of mitotic spindles and centrosomes stained with anti-α-tubulin mouse antibody, anti-pericentrin rabbit antibody, and Hoechst stain (DNA).
B). Moreover, radial arrays of microtubules emanated from all spindle poles, whether or not centrosomes were present (Fig. 3C and D). We note further that many of these bipolar spindles in MLN8054-treated cells show chromosome alignment defects. Given that these cells were treated with MLN8054 for only 5 h, the centrosome and spindle pole defects observed occurred within a single mitotic episode, and not subsequent to previous abnormal divisions.

To further establish the relationship between unseparated centrosomes and spindle poles in MLN8054-treated cells, centrosomes and spindle poles were quantified in mitotic cells from the above experiment (Fig. 4) as the number of distinct pericentrin- and NuMA-positive spots, respectively. As expected, nearly all control-treated cells presented with two centrosomes and two spindle poles per cell. Of the MLN8054-treated cells, 66% presented with only one centrosome, whereas only 11% contained only one spindle pole. The majority of MLN8054-treated cells formed more than one spindle pole. Of these, the majority were bipolar, but tri- and tetrapolar spindles were also observed. When centrosomes are present, they act as the dominant sites for microtubule nucleation. Thus, a cell with two separated centrosomes must contain at least two functional spindle poles, as defined by tubulin staining. Based on this premise, we may infer that 83% of the cells containing a single centrosome or unseparated centrosomes are capable of organizing one or more acentrosomal spindle poles.

Centrosome amplification in the presence of MLN8054 increases over time. The overexpression of both wild-type and kinase-dead Aurora A constructs in Chinese hamster ovary cells induced centrosome amplification through defects in cell division (38). We thus sought to test the effect of Aurora A inhibition by MLN8054 on centrosome number in mitotic cells over time. The number of centrosomes was assessed by using pericentrin in HCT-116 cells treated with DMSO or 0.25 μM MLN8054 for 5, 24, or 48 h and bortezomib for the final 2 h. Control-treated cells primarily contained two centrosomes, whereas cells treated with MLN8054 for 24 h occasionally displayed three centrosomes per mitotic cell (Fig. 5A). The number of cells with more than two centrosomes rose steadily, from 0% at 5 h to 9% at 24 h and 14% at 48 h (Fig. 5B). The increase in centrosome amplification with time upon Aurora A inhibition using MLN8054 is consistent with a model in which centrosome amplification occurs due to defects in previous cell divisions.

MLN8054 induces chromosomal defects in mitosis, leading to aneuploidy. Cells treated with MLN8054 for 5 and 24 h display chromosomal defects in all stages of mitosis, including chromosome alignment defects in metaphase, lagging chromosomes in anaphase, and chromatin bridges in telophase (Fig. 6). Given these defects, we explored the manifestation of aneuploidy in MLN8054-treated cells over time. HCT-116 cells were treated with 0.25 μM MLN8054 for 24, 48, or 72 h, and 5 μM aphidicholin was added 16 h before fixation to trap cells in the G1 cell cycle phase. Synchronizing cells in G1 provided a standardized means to evaluate nuclear integrity and DNA content after cells progressed through mitosis in the presence of MLN8054. Interphase nuclei from cells treated with MLN8054

![Figure 4](image-url)
displayed a variety of defects in form, including micronucleation, binucleation, and multinucleation (Fig. 7A). The frequency of these defects, collectively characterized as grossly abnormal interphase nuclei, increased with time, reaching a maximum at approximately 35% of total cells.

As a second, independent measure of aneuploidy, the number of kinetochores per cell was quantified in G1 cells prepared as described above. In G1, cells have one kinetochore per chromosome; therefore, measuring kinetochores is reflective of chromosome content. We deemed this approach superior to flow cytometry for the purpose of quantifying aneuploidy, as interpretation of DNA profiles generated by flow cytometry can be complicated by fragmented DNA derived from apoptotic cells. Cells were stained for kinetochores, α-tubulin, and DNA, and the numbers of kinetochores per cell were determined using serial-section confocal immunofluorescent microscopy and image processing (Fig. 7C). Histograms plotting the distribution of kinetochores in control- or MLN8054-treated cells were generated (Fig. 7D). The distribution of kinetochores in control-treated cells remained essentially unchanged over time. The distribution of kinetochores in MLN8054-treated cells at 24 h overlapped that of control-
treated cells. However, at 48 and 72 h, there were large populations of cells that had dramatic increases in kinetochores per cell. In fact, the increased distribution in kinetochores per cell at 48 h was similar to previous findings demonstrating an increased distribution in the DNA content determined by flow cytometry 48 h after the addition of MLN8054 (35). Interestingly, there was a peak of cells with a complement of kinetochores approximately twofold more that of the untreated cells, suggesting that these cells may have failed to complete cytokinesis prior to exiting mitosis and doubled their DNA content in the subsequent S phase. This is consistent with the results of previous reports demonstrating that perturbation of Aurora A can lead to a low incidence of cytokinesis failures (36). There were not a significant number of cells with a kinetochore-per-cell distribution below the range for kinetochores per cell of the control-treated samples. This suggests that cells with a suboptimal complement of DNA cannot survive and is consistent with previous reports describing massive chromosomal loss leading directly to cell death (31).

Overall, the findings described in this study demonstrate that inhibition of Aurora A by using MLN8054 leads to chromosome segregation defects that, in turn, cause severe aneuploidy over time.

**DISCUSSION**

The results presented in this paper put forth a model of the mechanism of action of the novel small-molecule inhibitor
FIG. 7. Aurora A inhibition leads to aneuploidy. HCT-116 cells were treated with DMSO (control) or 0.25 μM MLN8054 for 24, 48, or 72 h. A 5 μM concentration of aphidicholin was added for the final 16 h to arrest cells in the G1 phase of the cell cycle. (A) Representative immunofluorescent images of interphase cells stained with anti-α-tubulin rabbit antibody (green) and Hoechst stain (DNA, blue). Three example MLN8054-treated cells are shown. The yellow arrows indicate binucleated nuclei of various sizes. The scale bars represent 10 μm. (B) Percentage of interphase cells containing grossly abnormal nuclei. The P values demonstrate statistical differences between control- and MLN8054-treated cells in the percentages of cells with abnormal nuclei 24, 48, and 72 h after treatment. (C) Representative immunofluorescent images of control- and MLN8054-treated cells stained with anti-α-tubulin rabbit antibody (green), nuclear ANA-centromere autoantibody (kinetochores, red), and Hoechst stain (DNA, blue). (D) Number of kinetochores per cell in control- and MLN8054-treated cells 24, 48, and 72 h after treatment.
MLN8054 that is consistent with, and extends our understanding of, the loss of Aurora A function (Fig. 8). Briefly, this mechanism of action involves the initial development of abnormally formed monopolar, bipolar, or multipolar mitotic spindles. Acentrosomal spindle formation, in particular, occurs at a high frequency, as many of these cells contain unseparated centrosomes. With time, monopolar and multipolar spindles may resolve to form two opposing spindle poles, although some of these defects may lead immediately to cell death through defective mitoses. While spindle defects resulting from Aurora A inhibition induce mitotic delays, presumably through activation of the spindle assembly checkpoint, cells ultimately divide at a frequency near that of untreated cells. Aurora A inhibition leads to chromosome congression defects at metaphase, lagging chromosomes in anaphase, and telophase chromatin bridges. Cells treated with MLN8054 ultimately become aneuploid, which leads to death. The solid arrows within this model represent steps supported by evidence presented in this work.

FIG. 8. Proposed model of the mechanism of action for MLN8054, a small-molecule inhibitor of Aurora A. Chromosomes are represented in blue, kinetochores in red, mitotic spindles in green, and centrosomes in yellow. The inhibition of Aurora A leads to the formation of monopolar, bipolar, and multipolar spindles, often with unseparated centrosomes. Monopolar and multipolar spindles may resolve to form bipolar spindles or may die directly from defective mitoses. Although mitotic spindle defects lead to mitotic delays, many cells divide, at an incidence near that of untreated cells. Aurora A inhibition leads to chromosome congression defects at metaphase, lagging chromosomes in anaphase, and telophase chromatin bridges. Cells treated with MLN8054 ultimately become aneuploid, which leads to death. The solid arrows within this model represent steps supported by evidence presented in this work.

As a result of Aurora A inhibition with MLN8054, cells develop spindle abnormalities at a high frequency and often present as either monopolar or multipolar, consistent with the results of previous reports on Aurora A inhibition in a variety of experimental systems (6, 21, 36). From the use of a small-molecule inhibitor, however, it is clear that these outcomes are kinase activity dependent, as MLN8054 inhibits Aurora A enzymatic activity but does not affect protein abundance or localization (35). Spindle monopolarity that occurs subsequent to Aurora A loss of function results from failed centrosomal separation (6, 21). However, our data argue that many monopolar spindles resulting from unseparated centrosomes eventually form acentrosomal spindle poles, resulting in either bipolar or multipolar spindles. The results of a variety of studies of numerous cell types suggest that both centrosomal and acentrosomal pathways of spindle organization operate in the same cell type (reviewed in references 9, 13, and 48). We found that, 5 h after MLN8054 treatment, approximately 83% of cells with unseparated centrosomes are capable of organizing one or more acentrosomal spindle poles. This number is broadly consistent with an earlier finding that 80% (12/15) of bipolar spindles in cells microinjected with Aurora A antibodies showed centrosomes at only one spindle pole (36).

In addition to the resolution of monopolar spindles into bipolar spindles, it is possible that some multipolar spindles also resolve over time into bipolar spindles. This would suggest that the microtubule motor-driven mechanisms involved in acentrosomal spindle formation, which involve NuMA, dynein, and dynactin, may resolve spindle multipolarity, most likely via a process analogous to centrosomal clustering (44). Alternatively, multipolar spindles at metaphase may progress to multipolar anaphases; however, such cells likely die immediately from these defective divisions (46). We did observe a small number of multipolar anaphases in our experiments (data not shown).

Our study shows that cells treated with MLN8054 that are able to assemble bipolar spindles appear to do so with the presence of a variety of chromosome alignment and segregation defects. We show the presence of metaphase alignment defects, lagging chromosomes at anaphase, and telophase bridges. These findings are consistent with those of earlier studies in mammalian cells showing chromosome alignment
defects upon the perturbation of Aurora A function in a number of different experimental contexts. These earlier studies included antibody microinjection and the use of siRNA (36), as well as transfection with wild-type or mutant versions of Aurora A that were either kinase dead or unable to bind to protein phosphatase 1 (2, 28).

We further demonstrate that cells treated with MLN8054 develop an abnormal DNA content, as evidenced by the detection of abnormal nuclei formation and by immunofluorescence quantification of kinetochores. This result is consistent with the results of earlier studies using siRNA, as well as the results reported for antibody microinjection into cells in late G2 (36). Abnormalities in DNA content upon treatment with MLN8054 become more pronounced with time. Since MLN8054 inhibition of Aurora A kinase occurs as cells enter mitosis, we may infer that this outcome is due to an unequal segregation of DNA after one or more passages through mitosis.

Another possible outcome for cells derived from divisions of mitotic cells with unseparated centrosomes is the evolution of supernumerary centrosomes in subsequent cell cycles following centrosome duplication. In fact, prolonged treatment of cells with MLN8054 results in an increase in supernumerary centrosomes, an effect that becomes more pronounced with time. This is consistent with other findings on Aurora A perturbation, including those using a small-molecule inhibitor or Aurora A overexpression (20, 52).

The phenotypes we observe upon Aurora A inhibition using MLN8054 closely resemble those associated with the inhibition of other proteins involved in spindle assembly and chromosome congression (reviewed in references 30, 34, 45, and 49). While the phenotypic resemblance to at least some of these cases could well be coincidental, functional interactions between Aurora A and several of these proteins are known. For example, Drosophila melanogaster and Xenopus Aurora A both phosphorylate TACC3, which localizes TACC3 to centrosomes in early mitosis (3, 5, 16, 29, 43). This leads to a formation between TACC3 and the microtubule-associated protein XMAP215/ch-TOG, which facilitates microtubule growth (14, 33). Mutations in either TACC3 or XMAP215/ch-TOG also result in spindle and chromosome congression defects.

From the observation that MLN8054-treated cells undergo anaphase despite incomplete chromosome congression to the metaphase plate, we may infer a role for Aurora A in the maintenance of the spindle assembly checkpoint. Such an inference is plausible, given that direct connections between Aurora A and spindle assembly checkpoint components have been described. For example, Aurora A phosphorylates CENP-A on Ser7, which is necessary for the complete localization of Aurora B to kinetochores (32). Perturbations of Aurora B prevent the proper assembly of the spindle assembly checkpoint complex at kinetochores (11, 47). Further evidence supporting a role for Aurora A in spindle assembly checkpoint maintenance was demonstrated in the results of Aurora A overexpression studies (2, 27, 38).

There have been several reports describing other small-molecule inhibitors of the Aurora kinases (11, 12, 20, 23, 24). These inhibitors selectively inhibit Aurora B or are dual Aurora A/Aurora B inhibitors. All of these molecules potently inhibit the phosphorylation of histone H3 on Ser10, a specific Aurora B substrate in cells. Moreover, these molecules cause cells to quickly exit mitosis without undergoing anaphase or cytokinesis, which results from an inability to establish and maintain a spindle assembly checkpoint-mediated arrest prior to the alignment of chromosomes at the metaphase plate. Thus, the outcomes achieved in cells in response to these molecules are distinct from those observed in our studies using MLN8054 at concentrations selective for Aurora A.

The model presented in this paper puts forth a mechanism of action for Aurora A inhibition using the selective small-molecule inhibitor MLN8054 that is consistent with a chromosomal instability phenotype driven by severe chromosome alignment and segregation defects during mitosis. As MLN8054 induces robust tumor growth inhibition in cultured tumors and in human tumor xenographs (35), it is likely that Aurora A inhibition kills tumor cells through the development of deleterious aneuploidy.

ACKNOWLEDGMENTS

We are grateful to the Departments of Medicinal Chemistry and Discovery Technologies at Millennium Pharmaceuticals for the support they provided towards this work and to Benjamin Amidon, Katherine Galvin, Mark Manfredi, and Ole Petter Veiby for critical reading of the manuscript.

This work was supported by Millennium Pharmaceuticals, Inc.

REFERENCES

histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis. J. Cell Biol. 152:669–682.


