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Determinants for the efficiency of anticancer drugs targeting either Aurora-A or Aurora-B kinases in human colon carcinoma cells

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Abstract

The mitotic Aurora kinases, including Aurora-A and Aurora-B, are attractive novel targets for anticancer therapy, and inhibitory drugs have been developed that are currently undergoing clinical trials. However, the molecular mechanisms how these drugs induce tumor cell death are poorly understood. We have addressed this question by comparing the requirements for an efficient induction of apoptosis in response to MLN8054, a selective inhibitor of Aurora-A, and the selective Aurora-B inhibitor ZM447439 in human colon carcinoma cells. By using various isogenic knockout as well as inducible colon carcinoma cell lines, we found that treatment with MLN8054 induces defects in mitotic spindle assembly, which causes a transient spindle checkpoint–dependent mitotic arrest. This cell cycle arrest is not maintained due to the activity of MLN8054 to override the spindle checkpoint. Subsequently, MLN8054-treated cells exit from mitosis and activate a p53-dependent postmitotic G_1 checkpoint, which subsequently induces p21 and Bax, leading to G_1 arrest followed by the induction of apoptosis. In contrast, inhibition of Aurora-B by ZM447439 also interferes with normal chromosome alignment during mitosis and overrides the mitotic spindle checkpoint but allows a subsequent endoreduplication, although ZM447439 potently activates the p53-dependent postmitotic G_1 checkpoint. Moreover, the ZM447439-induced endoreduplication is a prerequisite for the efficiency of the drug. Thus, our results obtained in human colon carcinoma cells indicate that although both Aurora kinase inhibitors are potent inducers of tumor cell death, the pathways leading to the induction of apoptosis in response to these drugs are distinct. [Mol Cancer Ther 2009;8(7):2046–56]

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Introduction

Targeting the progression of mitosis is a highly successful strategy for anticancer treatment (1). Recently, much attention has been drawn to the Aurora kinases, which comprise three family members, Aurora-A, Aurora-B, and Aurora-C, as novel mitotic drug targets. At least Aurora-A and Aurora-B function as key regulators of mitosis and they are frequently overexpressed in human cancer (2–5), which provides the basis for their importance as chemotherapeutic drug targets.

Aurora-A is localized on duplicated centrosomes and spindle poles during mitosis and is required for the timely entry into mitosis and proper formation of a bipolar mitotic spindle by regulating centrosome maturation, separation, and microtubule nucleation activity (6). In contrast, Aurora-B is a chromosomal passenger protein, which is, together with INCENP, borealin, and survivin, part of the chromosomal passenger complex (7). This complex changes its localization during mitotic progression from centromeres in early mitosis to the spindle midzone in anaphase and finally to the cleavage furrow and the midbody during cytokinesis. According to the different localizations, Aurora-B is required for phosphorylation of histone H3 (8), the proper biorientation and alignment of chromosomes by correcting faulty microtubule-kinetochore attachments (9, 10), and the execution of cytokinesis (11). It has also been suggested that Aurora-B might contribute to spindle checkpoint function, which monitors proper chromosome alignment during mitosis (9, 12), although it is not clear whether this role is related to its function to resolve incorrect kinetochore attachments. The third member of the Aurora kinase family, Aurora-C, might have functions during meiosis rather than during mitosis (13) and it seems not to be aberrantly expressed in human cancer (2).

Extensive efforts have been made to develop inhibitors for Aurora-A and Aurora-B, and several highly potent inhibitory compounds have been identified. Examples are Hesperadin (10), ZM447439 (9), and AZD1152 (14), which show selectivity for Aurora-B *in vivo* and VX-680 (MK-0547), which inhibits both Aurora-A and Aurora-B (15, 16). Most recently, the first Aurora-A selective inhibitor, MLN8054, has been introduced (17). It has been shown that treatment of cells with Hesperadin, ZM447439, AZD1152, or VX-680 inhibits the phosphorylation of histone H3 and cytokinesis and induces polyploidization (9, 10, 14), indicating that these drugs inhibit Aurora-B *in vivo*. In contrast, VX-680 and MLN8054 treatment abolishes the activation-specific

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autophosphorylation of Aurora-A on Thr^{288} (16, 17). At the same time, MLN8054 treatment leaves the histone H3 phosphorylation intact (17). For some inhibitors (e.g., AZD1152, VX-680, and MLN8054), an efficacy *in vivo* on human tumor xenografts has been shown and several compounds are currently investigated in clinical trials (1).

Thus far, little is known about how Aurora inhibition causes tumor cell death. In fact, a detailed knowledge of the molecular pathways involved in the induction of apoptosis in response to inhibition of either Aurora-A or Aurora-B is essential to improve therapeutic strategies, to circumvent drug resistance, and to answer the long-standing question about what the better target is, Aurora-A or Aurora-B. Here, we used the pharmacologic inhibitors MLN8054 and ZM447439 that selectively target the Aurora-A or Aurora-B kinases, respectively. To define the molecular pathways that are activated and required for an efficient induction of apoptosis after treatment with these drugs, we took advantage of the well-established and genetically defined HCT116 human colon carcinoma cell system and a set of different isogenic knockout derivatives thereof.

Materials and Methods

Cell Culture

HCT116, HCT-*p53*$^{-/-}$, HCT-*p21*$^{-/-}$ (18), HCT-*BAX*$^{-/-}$ (19), and HCT-*MAD2*$^{+/-}$ (20) cell lines were grown in RPMI 1640, 10% FCS, 1% glutamine, 100 μg/mL streptomycin, and 100 units/mL penicillin (Invitrogen). RKO-p21 and RKO-p27 cells (21) were maintained in DMEM plus 10% FCS, 500 μg/mL G418, and 200 μg/mL zeocin (Invitrogen). *p21* and *p27* expression was induced by addition of 10 μmol/L ponasteron-A (Sigma). RKO cells were synchronized at G_1-S by treatment with 1 μg/mL aphidicolin (Alexis) for 24 h followed by release into medium.

Drug Treatments

Cells were treated with 0.1 to 2.0 μmol/L of ZM447439 (Biomol), 0.1 to 2.0 μmol/L of MLN8054 (a kind gift from Millennium Pharmaceuticals, Inc.), 68 μmol/L monastrol (Biomol), 300 nmol/L nocodazole (Sigma), 100 nmol/L Taxol (Sigma), and 20 μmol/L MG132 (Calbiochem).

Flow Cytometry

Harvested cells were fixed in 70% (v/v) ethanol at 4°C for 16 h. The DNA content was determined by staining cells with 50 μg/mL propidium iodide (Sigma). The mitotic index was determined by doing intracellular staining with anti-MPM2 antibodies (Upstate) as described before (22). Apoptotic cells were identified as cells with a sub-G_1 DNA content. Cells were analyzed on a FACSCalibur (Becton Dickinson) and data analysis was carried out using the CellQuest Pro software.

Western Blotting

Cell lysates were prepared in lysis buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 1% (v/v) NP40, 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 20 mmol/L sodium orthovanadate, 0.5 μmol/L microcystin, complete protease inhibitors (Roche)]. SDS-PAGE and semidry Western blotting and detection were done using standard protocols. The following antibodies were used for Western blotting: anti–Aurora-A (Santa Cruz Biotechnology), anti–Aurora-A-pT288 (Cell Signaling), anti–Aurora-B (BD Transduction Laboratories), anti–Aurora-B-pT232 (Cell Signaling), anti-actin (Sigma), anti–cyclin B (Santa Cruz Biotechnology), anti-securin (Lab Vision), anti-Bub1 (a gift from Stephen Taylor, University of Manchester, Manchester, UK), anti-BubR1 (Chemicon), anti-Bub3 (BD Transduction Laboratories), anti-p53 (Oncogene), anti-p21 (Oncogene), anti-p27 (Santa Cruz Biotechnology), and anti-Bax (Cell Signaling). Secondary antibodies conjugated to horseradish peroxidase were from Jackson. The enhanced chemiluminescence system was used for detection.

Microscopy

Cells were grown on poly-lysine–coated coverslips and fixed in 2% paraformaldehyde for 5 min followed by permeabilization in methanol at −20°C. Primary antibodies [α-tubulin (Sigma), CREST (Europa Bioproducts), phospho-histone H3 (Ser^{10}; Cell Signaling), Bub1, and BubR1] were incubated for 2 h at room temperature followed by a 2-h incubation with Alexa Fluor–conjugated secondary antibodies (Molecular Probes). DNA was stained with 4′,6-diamidino-2-phenylindole and microscopy was carried out using a Leica DM6000B microscope. Z-optical stacks with a spacing of 0.2 μm were recorded, and deconvolution of images and quantitation of fluorescence intensities were carried out using the Leica LAS-AF software.

Results

MLN8054 Is a Specific Inhibitor for Aurora-A and ZM447439 Inhibits Aurora-B Selectively in Human Colon Carcinoma Cells

To determine the specific requirements for the efficacy of Aurora kinase inhibitors in human colon carcinoma cells, we selected MLN8054 and ZM447439. MLN8054 represents a compound that displays a high selectivity for Aurora-A over Aurora-B (IC_{50}: 4 and 172 nmol/L, respectively; ref. 17), whereas ZM447439 shows a clear selectivity for Aurora-B over Aurora-A (IC_{50}: 50 and 1,000 nmol/L, respectively; ref. 23). The selectivity of these drugs in our experimental system based on HCT116 cells was verified by analyzing the autophosphorylation status within the activation loops of Aurora-A and Aurora-B on Western blots (Fig. 1A), by evaluating the Aurora-B–mediated phosphorylation of histone H3 on Ser^{10} in mitotic cells in immunofluorescence studies (Supplementary Fig. S1A),[1] and by investigating the inhibition of cytokinesis that is dependent on Aurora-B by determining the DNA content in fluorescence-activated cell sorting (FACS) analyses (Supplementary Fig. S1B).[1] All these cellular assays confirmed the selectivity of the drugs for Aurora-A or Aurora-B when used at 0.5 and 2 μmol/L, respectively.

[1] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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In addition, we analyzed the formation of the mitotic spindle after releasing cells from a monastrol block in the presence or absence of the drugs (Supplementary Fig. S1C).[1] In agreement with previous results, inhibition of Aurora-A inhibits bipolar mitotic spindle assembly, whereas inhibition of Aurora-B prevents the correct microtubule-kinetochore attachment causing severe chromosome alignment defects (9, 10, 17, 23). Together, these results verify that MLN8054 and ZM447439 are indeed well suited to discriminate between inhibition of Aurora-A and Aurora-B in our experimental cell system.

Inhibition of Aurora-A, but not of Aurora-B, Causes a Mitotic Arrest That Cannot Be Maintained

When we treated asynchronously growing HCT116 cells with different concentrations of MLN8054 or ZM447439, an accumulation of mitotic cells was only apparent after MLN8054 but not on ZM447439 treatment (Fig. 1B, *left*), and as expected, this mitotic accumulation was associated with the generation of monopolar and aberrant spindle structures (Fig. 1B, *right*). Then, we investigated whether the MLN8054-induced mitotic delay is dependent on the mitotic spindle checkpoint. We treated HCT116 and isogenic spindle checkpoint–defective cells harboring a deletion of one allele of *MAD2* (HCT-*MAD2*$^{+/-}$; refs. 20, 22) with nocodazole or MLN8054 and found that a functional spindle checkpoint is required for both the nocodazole- and MLN8054-induced mitotic delay (Supplementary Fig. S2).[1]

Because MLN8054 treatment strongly induces the formation of monopolar spindles, we compared the effects of MLN8054 with another antimitotic drug that causes the generation of monopolar spindles, namely, the KSP/Eg5 kinesin inhibitor monastrol (24). Asynchronously growing HCT116 cells were treated with either monastrol or MLN8054, and the mitotic accumulation was followed over time. Interestingly, although both drugs inhibit the formation of bipolar spindles, monastrol treatment caused a much higher mitotic index than MLN8054 treatment (Fig. 1C). Moreover, when analyzing the DNA content by FACS analyses, we found that monastrol treatment led to an accumulation of cells with 4N content, whereas MLN8054 treatment causes only transiently an accumulation of cells with a 4N DNA content but allows cell division for a subset of cells generating cells with a 2N DNA content over time (Fig. 1D), which is consistent with previous findings indicating that MLN8054-treated cells can ultimately divide (25). Together, our results show that MLN8054 treatment leads to activation of the mitotic spindle checkpoint associated with a transient

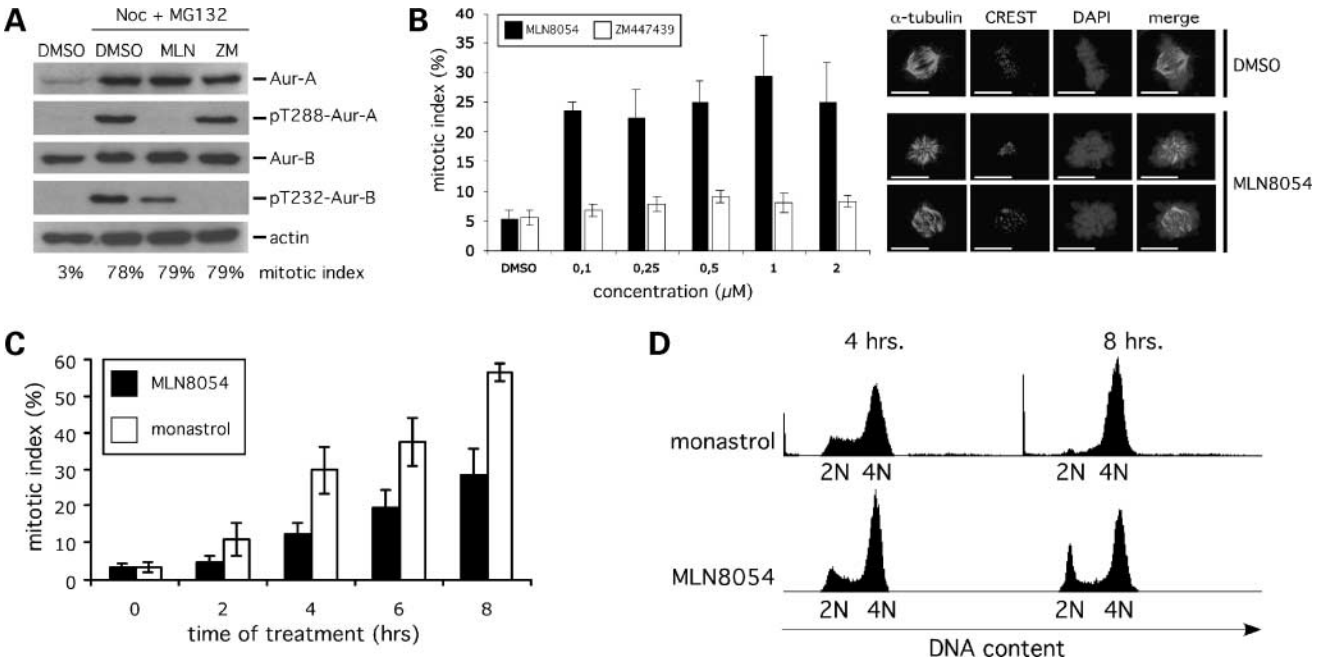

Figure 1. MLN8054 but not ZM447439 treatment induces a spindle checkpoint–mediated mitotic delay that cannot be maintained. **A,** selectivity of MLN8054 and ZM447439 for inhibition of Aurora-A and Aurora-B, respectively. Human colon carcinoma (HCT116) cells were treated with nocodazole for 14 h followed by addition of the proteasome inhibitor MG132 and either DMSO, 0.5 μmol/L MLN8054, or 2 μmol/L ZM447439 for additional 2 h. Aurora-A and Aurora-B proteins phosphorylated on Thr^{288} or Thr^{232}, respectively, were detected on Western blots. **B,** MLN8054 induces a transient mitotic arrest with aberrant mitotic spindles. *Left,* quantitation of drug-induced mitotic arrest. HCT116 cells were treated with various concentrations of either MLN8054 or ZM447439 for 8 h and the mitotic index was determined. *Right,* MLN8054 induces aberrant mitotic spindles. Cells were treated with 0.5 μmol/L MLN8054 for 16 h, fixed, and stained for microtubules (*green*, α-tubulin) and kinetochores (*red*, CREST). DNA was stained with 4′,6-diamidino-2-phenylindole (*DAPI*; *blue*). Representative examples of mitotic cells are shown. Scale bar, 10 μm. **C,** comparison of the MLN8054- and monastrol-induced mitotic delay. HCT116 cells were treated with 0.5 μmol/L MLN8054 or 68 μmol/L monastrol for up to 8 h and the mitotic index was determined. **D,** MLN8054 treatment allows exit from mitosis. Cells were treated as in **C** and representative examples of FACS profiles of cells treated with monastrol or MLN8054 for 4 and 8 h are shown. *Columns,* mean from at least three independent experiments; *bars,* SD.

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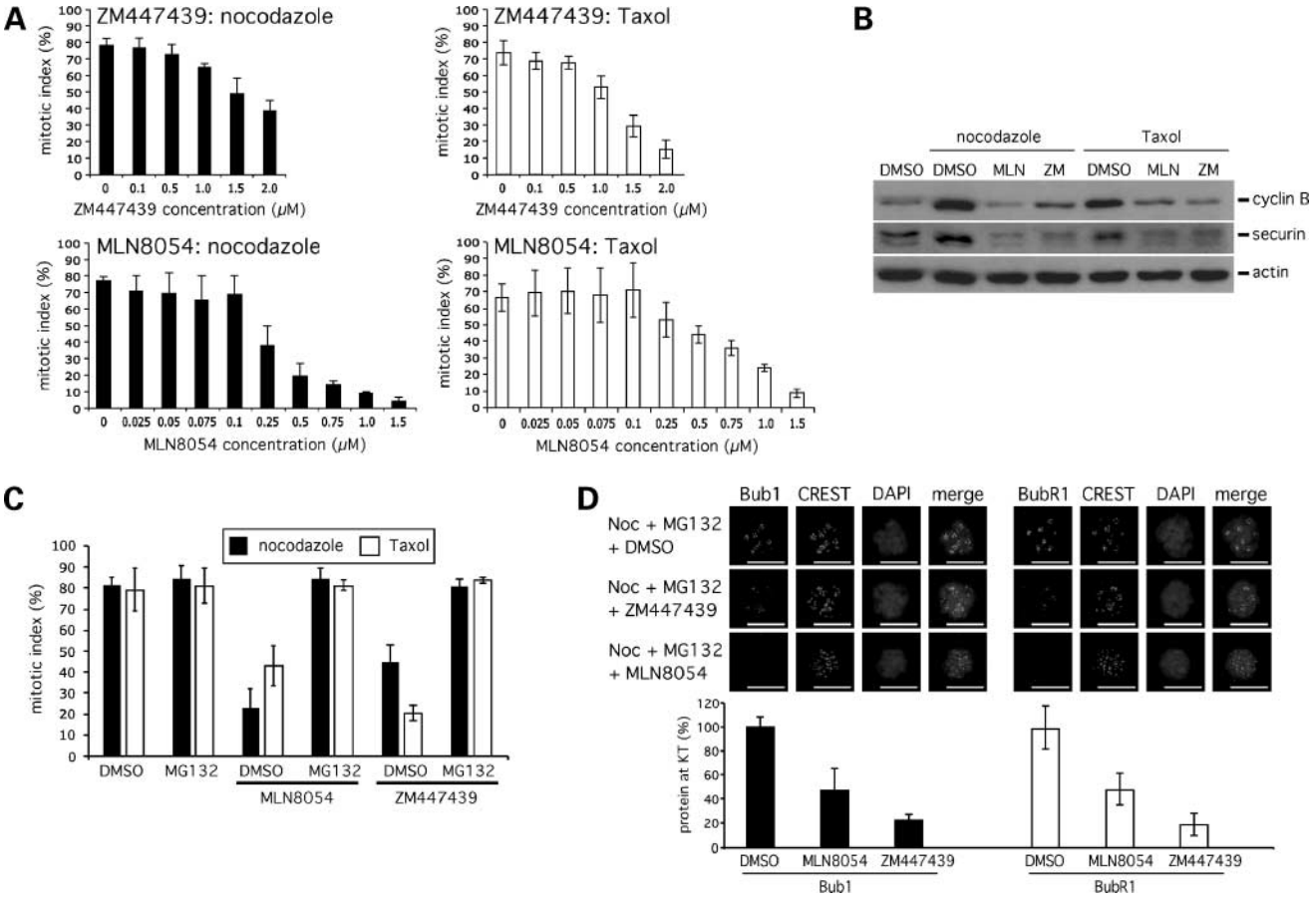

Figure 2. MLN8054 and ZM447439 treatment can override a spindle checkpoint–mediated mitotic arrest. **A,** ZM447439 (*top*) and MLN8054 (*bottom*) override a nocodazole- and Taxol-induced mitotic arrest. HCT116 cells were treated with 300 nmol/L nocodazole (*left*) or 100 nmol/L Taxol (*right*) for 14 h followed by addition of different concentrations of ZM447439 or MLN8054 for additional 2 h and the mitotic index was determined. **B,** MLN8054 or ZM447439 treatment induces mitotic proteolysis of cyclin B and securin. Cells were treated as in **A** and **B** and the protein level of cyclin B, securin, and actin was determined on Western blots. **C,** the MLN8054- or ZM447439-induced mitotic exit is suppressed by proteasome inhibition. Cells were treated with nocodazole or Taxol for 14 h followed by treatment with 0.5 μmol/L MLN8054 or 2 μmol/L ZM447439 in the presence or absence of the proteasome inhibitor MG132 and the mitotic index was determined after 2 h. **D,** detection and quantitation of kinetochore-localized Bub1 and BubR1 proteins in response to MLN8054 and ZM447439 treatment. Cells were treated with nocodazole for 8 h followed by addition of DMSO, 0.5 μmol/L MLN8054, or 2 μmol/L ZM447439 in the presence of MG132 to prevent exit from mitosis. Bub1 and BubR1 proteins (*green*) were localized by immunofluorescence microscopy and kinetochore localization was verified by colocalization with CREST signals (*red*). *Blue,* mitotic chromosomes are stained with 4′,6-diamidino-2-phenylindole. Scale bar, 10 μm. Fluorescence intensities were quantified relative to the CREST signal. For each quantitation, at least 80 kinetochores from six cells were analyzed. *Columns,* mean from at least three independent experiments; *bars,* SD.

mitotic arrest, which cannot be maintained over time, allowing an unscheduled exit from mitosis and the generation of cells with a 2N DNA content.

Both MLN8054 and ZM447439 Override a Spindle Checkpoint–Mediated Mitotic Arrest

The inability of MLN8054 to induce a stable mitotic arrest in response to the formation of monopolar and aberrant mitotic spindles might suggest that MLN8054 can override the spindle checkpoint. To directly test whether MLN8054 can override an activated spindle checkpoint, we perform dose-response experiments with MLN8054 and ZM447439 on HCT116 cells treated with nocodazole or Taxol to activate the spindle checkpoint and to arrest cells in mitosis. As shown previously, ZM447439 could override the mitotic arrest imposed by Taxol treatment much more efficiently than after nocodazole treatment (IC_{50}: ~1.3 μmol/L versus ~2.0 μmol/L, respectively; Fig. 2A, *top*; refs. 9, 10). Significantly, MLN8054 was also able to override the spindle damage–induced mitotic arrest efficiently. In contrast to ZM447439, we found that MLN8054 is much more efficient in inactivating a nocodazole-induced than a Taxol-induced mitotic arrest (IC_{50}: ~0.25 μmol/L versus ~0.75 μmol/L, respectively; Fig. 2A, *bottom*). Because inactivation of the spindle checkpoint leads to reactivation of the anaphase-promoting complex/cyclosome and subsequent proteasome-mediated degradation of key mitotic regulators, including cyclin B and securin (26), we determined those protein levels in response to MLN8054 or ZM447439 treatment in the presence of nocodazole or Taxol. Clearly, treatment with Aurora inhibitors in the presence of nocodazole or Taxol led to proteolysis of cyclin B and securin, and consistent with a more efficient inhibition of a

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nocodazole-induced mitotic arrest, MLN8054-treated cells displayed a lower level of cyclin B in the presence of nocodazole than Taxol (Fig. 2B). Moreover, the Aurora inhibitor–mediated escape from the mitotic arrest was suppressed by cotreatment with the proteasome inhibitor MG132 (Fig. 2C). This indicates that inhibition of Aurora-A or Aurora-B relieves the inhibition of the proteasome-dependent mitotic proteolysis machinery and supports our finding that Aurora kinase inhibitors can over-ride the spindle checkpoint.

Activation of the spindle checkpoint is associated with a recruitment of spindle checkpoint proteins to kinetochores that are not attached to microtubules or that are not under tension (26). We investigated the kinetochore localization of the spindle checkpoint proteins Bub1 and BubR1 after treatment of mitotic cells with MLN8054 or ZM447439 and

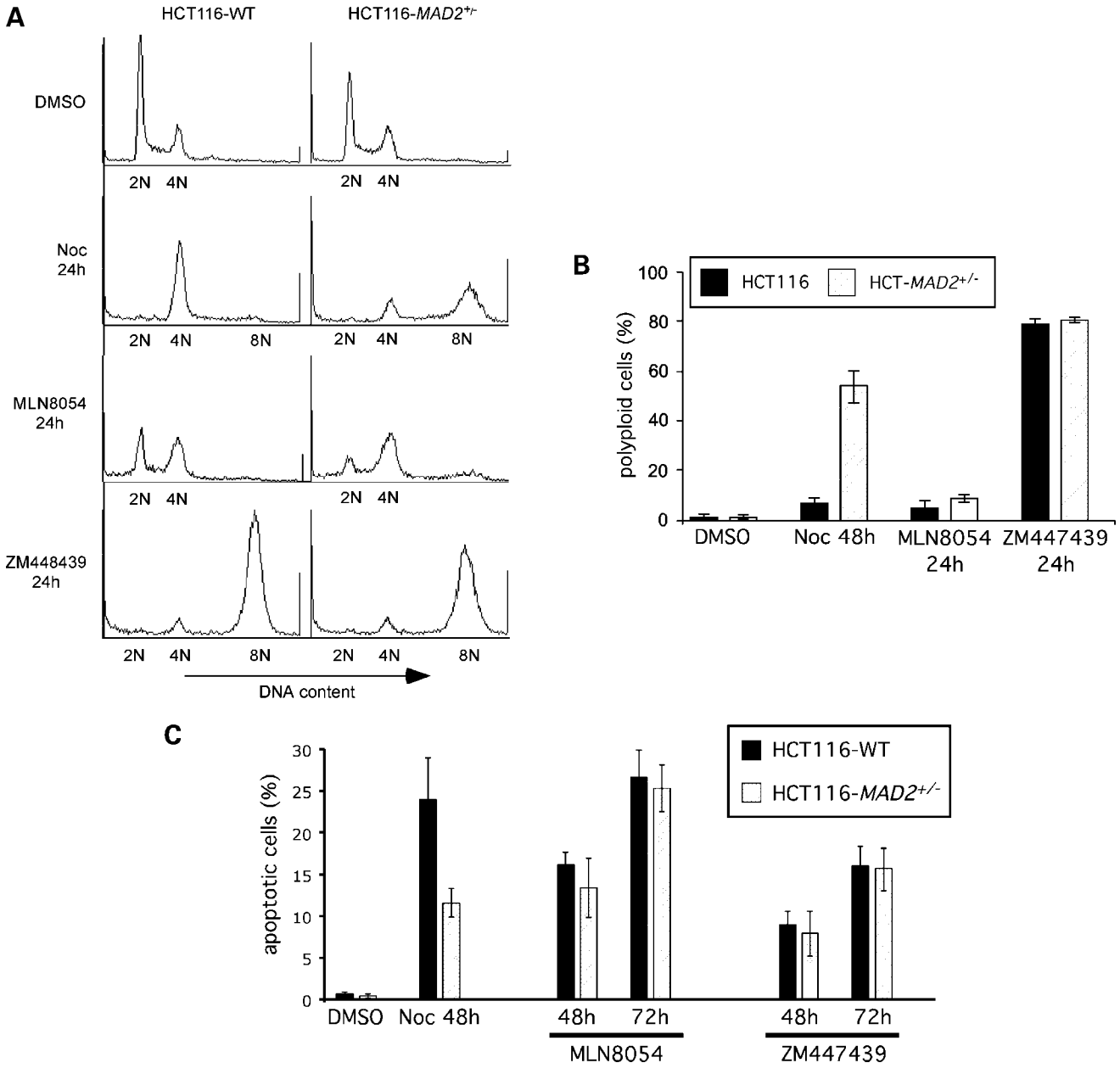

Figure 3. MLN8054 and ZM447439 induce apoptosis independent of a functional spindle checkpoint. **A,** the status of the mitotic spindle checkpoint does not determine the fate of cells treated with Aurora inhibitors. HCT116 and isogenic HCT-*MAD2*$^{+/-}$ cells were treated with 300 nmol/L nocodazole, 0.5 μmol/L MLN8054, or 2 μmol/L ZM447439 for 24 h and the DNA content was determined by FACS analyses. Examples of representative results are shown. **B,** quantitation of polyploidy (DNA content >4N) induced by nocodazole, MLN8054, and ZM447439 in HCT116 and HCT-*MAD2*$^{+/-}$ cells. **C,** determination of the proportion of apoptotic HCT116 or HCT-*MAD2*$^{+/-}$ cells after treatment with nocodazole, MLN8054, or ZM447439 for 48 and 72 h. *Columns,* mean from at least three independent experiments; *bars,* SD.

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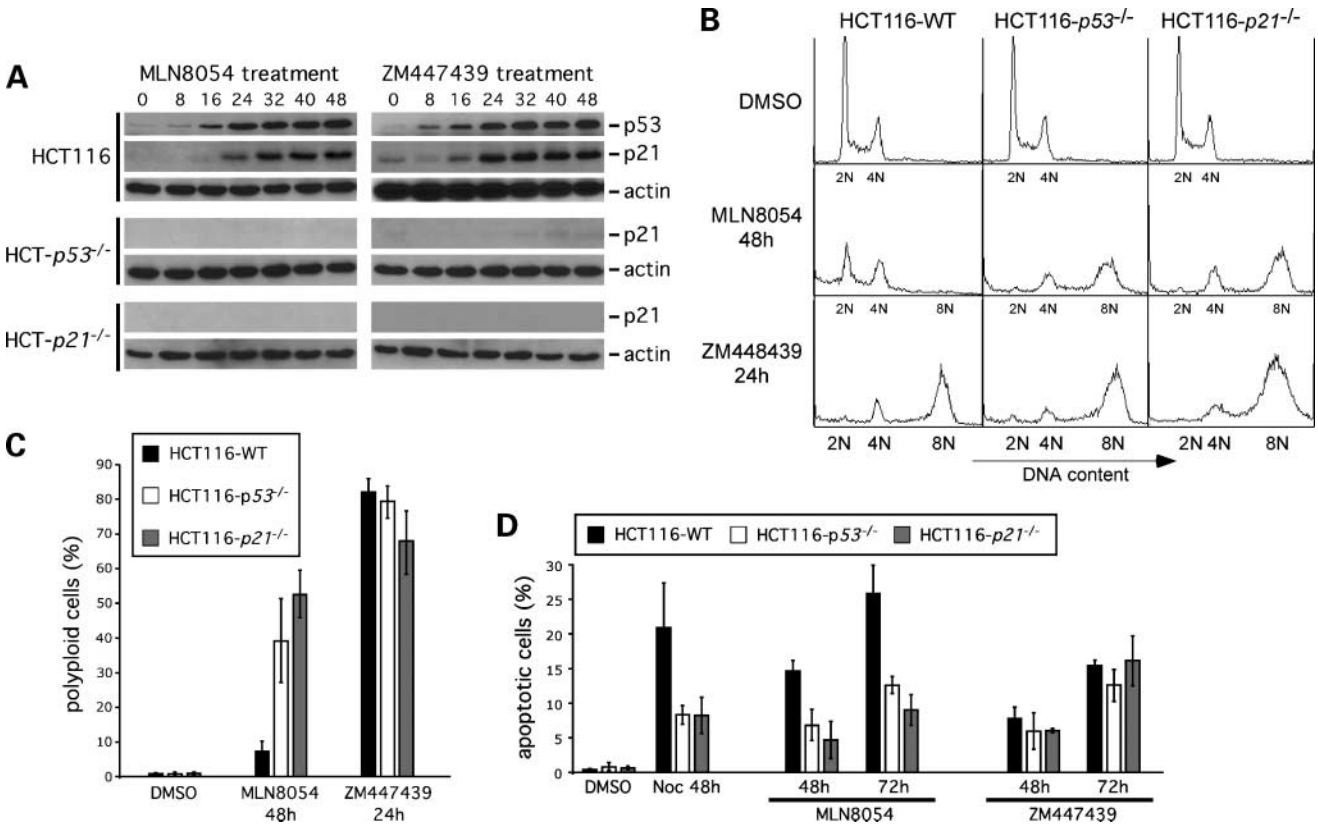

Figure 4. The integrity of the postmitotic G_1 checkpoint determines the fate and the induction of apoptosis after treatment with MLN8054 but not with ZM447439. **A,** activation of a p53-dependent postmitotic G_1 checkpoint by Aurora inhibitors. HCT116 cells were treated with 0.5 μmol/L MLN8054 or 2 μmol/L ZM447439 for up to 48 h and the protein level of p53, p21, and actin was determined on Western blots. **B,** HCT116 and isogenic p53- or p21-deficient derivative cell lines were treated with MLN8054 or ZM447439 and the DNA content was determined by FACS. Representative DNA profiles are shown. **C,** quantitation of polyploidy (DNA content >4N) in different HCT116 cell lines treated as described in **B**. **D,** determination of the proportion of apoptotic HCT116 or isogenic p53- or p21-deficient cells after treatment with nocodazole, MLN8054, or ZM447439. *Columns,* mean from at least three independent experiments; *bars,* SD.

found that treatment with both Aurora kinase inhibitors reduced the kinetochore localization of both spindle checkpoint proteins. Quantitation of the amount of Bub1 and BubR1 at kinetochores relative to a kinetochore marker (CREST) revealed a ~80% reduction of Bub1 and BubR1 in response to ZM447439 treatment and a ~60% reduction after MLN8054 treatment (Fig. 2D), whereas the overall level of the spindle checkpoint proteins was not impaired (Supplementary Fig. S3).[1] Thus, the reduced amount of spindle checkpoint proteins at kinetochores might explain the inhibition of the spindle checkpoint on treatment with either MLN8054 or ZM447439. The exact mechanism how the Aurora inhibitors can override the spindle checkpoint leading to loss of checkpoint proteins at kinetochores is currently not known and deserves further detailed analyses.

The Induction of Apoptosis on Aurora-A or Aurora-B Inhibition Does Not Require a Functional Spindle Checkpoint

Recent evidence has indicated that the induction of apoptosis in response to various antimitotic drug treatments is dependent on a functional spindle checkpoint (22, 27–30). Therefore, we investigated whether the efficiency of MLN8054 or ZM447439 relies on a functional spindle checkpoint. HCT116 and isogenic spindle checkpoint–compromised HCT-*MAD2*$^{+/-}$ cells were treated with nocodazole, MLN8054, or ZM447439 for up to 72 hours, and cell cycle profiles and the induction of cell death were determined. As shown previously and consistent with an impaired function of the spindle checkpoint, *MAD2*$^{+/-}$ cells exhibited a reduced mitotic arrest (Supplementary Fig. S2; ref. 20), severe endoreduplication (Fig. 3A and B; ref. 31), and reduced apoptosis (Fig. 3C; ref. 22) after prolonged treatment with nocodazole. Interestingly, HCT-*MAD2*$^{+/-}$ cells treated with MLN8054 showed a reduced mitotic arrest (Supplementary Fig. S2) but no endoreduplication (Fig. 3A and B) and no alteration in the rate of apoptosis (Fig. 3C). However, treatment with ZM447439 induced polyploidization in wild-type as well as in spindle checkpoint–impaired cells (Fig. 3A and B) and the induction of apoptosis was not dependent on a functional spindle checkpoint (Fig. 3C). Thus, a functional spindle checkpoint is not essential for the induction of cell death

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on treatment with the Aurora kinase inhibitors MLN8054 and ZM447439 in human colon carcinoma cells.

A p53-Dependent Postmitotic Checkpoint Is Required for the Induction of Apoptosis after MLN8054 but not after ZM447439 Treatment

On prolonged treatment with spindle poisons, cells can escape from mitosis and activate a postmitotic G_1 checkpoint, which arrests cells in a p53-dependent manner before S phase. This checkpoint is thought to act as a second fail-safe mechanism for cells that exited aberrantly from mitosis (31, 32). Because treatment with Aurora kinase inhibitors causes an aberrant progression and exit from mitosis, we investigated if this is associated with the activation of the postmitotic G_1 checkpoint. Indeed, treatment with MLN8054 or ZM447439 strongly activated p53 and induced p21 in a p53-dependent manner in our HCT116 cell system (Fig. 4A). Interestingly, the activation of the postmitotic checkpoint caused a G_1 arrest on MLN8054 but not after ZM447439 treatment (Fig. 4B and C). Moreover, p53 and p21 are required to maintain a postmitotic G_1 checkpoint triggered by MLN8054 but not after ZM447439 treatment. Isogenic HCT116 cells deficient for p53 or p21 (HCT-$p53^{-/-}$ and HCT-$p21^{-/-}$; ref. 18) exhibited severe endoreduplication after treatment with MLN8054, whereas ZM447439-mediated polyploidization was not significantly affected in the different knockout cell lines (Fig. 4B and C). Thus, both MLN8054 and ZM447439 activate a p53-dependent postmitotic G_1 checkpoint, but its genetic inactivation has only an effect on the fate of MLN8054-treated HCT116 cells.

To examine whether the postmitotic G_1 checkpoint is important for the induction of cell death after Aurora inhibition, we treated HCT116 and isogenic *TP53*- and *p21*-deficient cells with nocodazole, MLN8054, or ZM447439 and determined the proportion of apoptotic cells. Significantly, loss of either p53 or p21 protected cells from apoptosis after treatment with MLN8054 or nocodazole, whereas ZM447439-induced apoptosis was not altered (Fig. 4D). Thus, the p53-dependent postmitotic G_1 checkpoint is required for MLN8054-induced but not ZM447439-induced cell death, although both drugs strongly activate the postmitotic p53 response in human colon carcinoma cells.

Endoreduplication Is an Important Trigger for the Induction of Apoptosis after Aurora-B Inhibition

After treatment with ZM447439, polyploidization was not dependent on the p53-p21 status but correlated with the induction of apoptosis. Therefore, we evaluated whether endoreduplication is a prerequisite for the efficacy of ZM447439. At this point, we took advantage of previously characterized RKO colon carcinoma cell lines that express either the cyclin-dependent kinase inhibitor p21 (RKO-p21) or p27 (RKO-p27) in a ponasteron-inducible manner (21). Similar to the isogenic HCT116 cell system, the use of

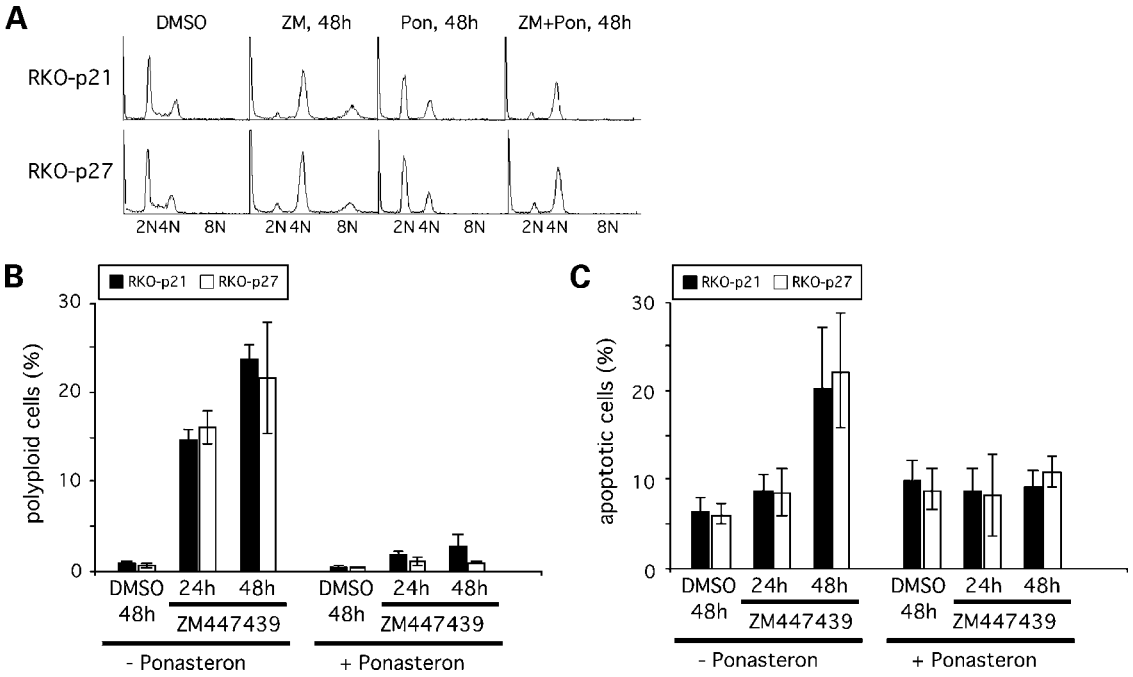

Figure 5. Drug-induced endoreduplication is required for ZM447439 efficacy. **A,** induced expression of p21 or p27 abolishes ZM447439-induced endoreduplication. RKO-p21 and RKO-p27 cells were synchronized at G_1-S by an aphidicolin block. Four hours after release, either DMSO or ZM447439 together with solvent or ponasteron-A was added and the DNA content was determined. Representative FACS profiles are shown. **B,** quantitation of ZM447439-induced polyploidy in the presence or absence of ponasteron-A from the experiments described in **A**. **C,** quantitation of ZM447439-induced apoptosis in the presence or absence of induction of p21 or p27. The proportion of apoptotic cells was determined after 48 and 72 h of ZM447439 treatment. *Columns,* mean from three independent experiments; *bars,* SD.

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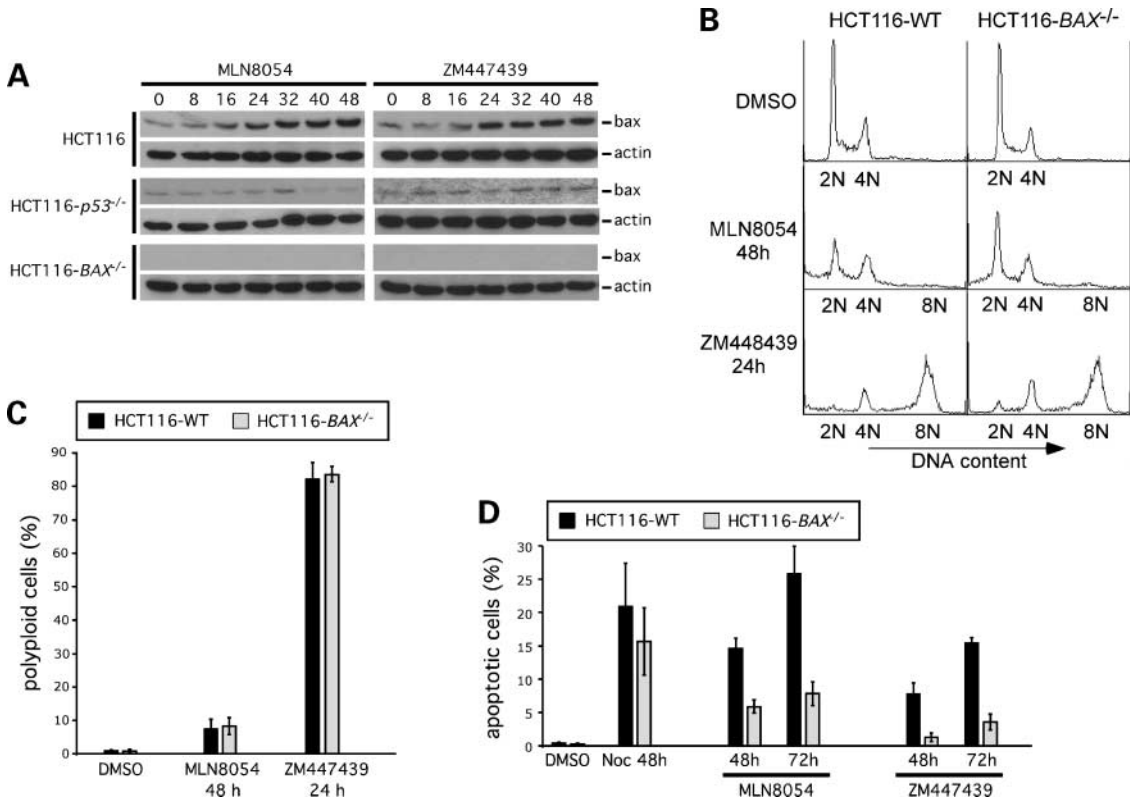

Figure 6. Bax is required for Aurora inhibitor–induced apoptosis. **A,** p53-dependent induction of *BAX* in response to MLN8054 and ZM447439 treatment. HCT116 and *p53*- or *BAX*-deficient derivative cell lines were treated with 0.5 μmol/L MLN8054 or 2 μmol/L ZM447439 and the protein level of Bax and actin was determined on Western blots. **B,** Bax does not determine the fate of cells after Aurora inhibitor treatment. HCT116 and isogenic *BAX*-deficient cells were treated with MLN8054 or ZM447439 and the DNA content was determined by FACS. Representative DNA profiles are shown. **C,** quantitation of polyploid HCT116 and HCT-$BAX^{-/-}$ cells after treatment with MLN8054 or ZM447439 for the indicated times. **D,** quantitation of MLN8054- and ZM447439-induced apoptosis in HCT116 and HCT-$BAX^{-/-}$ cells after treatment with the Aurora inhibitors for 48 and 72 h. *Columns,* mean from three independent experiments; *bars,* SD.

these inducible RKO colon carcinoma cells allows a defined phenotypic analysis in an isogenic genetic background. As expected, after addition of ponasteron-A, expression of *p21* or *p27* was induced and caused a cell cycle arrest in G_1 (Supplementary Fig. S4).[1] Both RKO cell lines were sensitive toward ZM447439 treatment and exhibited endoreduplication in response to ZM447439 treatment (Supplementary Fig. S5).[1] To test whether induced expression of either *p21* or *p27* would suppress the endoreduplication induced by ZM447439 treatment, cells were synchronized at G_1-S (Supplementary Fig. S6).[1] On release from the block, cells were treated with ZM447439 and ponasteron to induce the expression of p21 or p27 and endoreduplication and the induction of apoptosis were analyzed after progression through mitosis. Significantly, the induced expression of *p21* or *p27* in the presence of ZM447439 led to an accumulation of tetraploid cells and endoreduplication was suppressed (Fig. 5A and B). Importantly, the forced postmitotic G_1 arrest also protected RKO cells from ZM447439-induced apoptosis (Fig. 5C). Thus, the endoreduplication induced by ZM447439-mediated Aurora-B inhibition is an important trigger for the efficacy of the drug in these colon carcinoma cells.

Bax Is Required for the Induction of Apoptosis after MLN8054 or ZM447439 Treatment

The activation of p53 in response to drug treatment not only causes the induction of *p21* to mediate a cell cycle arrest but also triggers the induction of proapoptotic genes. *BAX* is such an important and well-established proapoptotic target gene of p53 and an important mediator of the intrinsic apoptotic pathway (33). We monitored the protein level of Bax in response to MLN8054 and ZM447439 treatment in HCT116 cells and found that *BAX* is expressed at a basal level but is strongly induced in a p53-dependent manner after treatment with either MLN8054 or ZM447439 (Fig. 6A). Further, we treated HCT116 and isogenic cells deficient for *BAX* (HCT-$BAX^{-/-}$) with MLN8054 or ZM447439 and analyzed both the rate of endoreduplication as a measure for the integrity of the postmitotic G_1 checkpoint and the induction of apoptosis. Clearly, Bax was neither required for the MLN8054-induced postmitotic G_1 arrest nor for ZM447439-induced endoreduplication (Fig. 6B and C). However, Bax was required for the induction of apoptosis after treatment with MLN8054 and ZM447439 but not after drug-induced spindle damage (Fig. 6D). The suppression of apoptosis in *BAX*-deficient cells after treatment with

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MLN8054 was associated with an enhanced survival of G_1-arrested cells (Fig. 6B), further supporting our result that the postmitotic G_1 arrest is required for the induction of apoptosis after MLN8054 treatment (Fig. 4).

Discussion

Aurora inhibitors target the mitotic functions of the Aurora kinases and induce apoptosis in cultured tumor cells as well as in human xenografts (2, 9, 10, 14, 15, 34–36). However, the molecular mechanisms leading to the induction of tumor cell death are poorly understood, although a detailed knowledge about these mechanisms is most important to improve therapeutic strategies and drug combinations and to explain resistance on a molecular level.

By the use of various isogenic somatic knockout as well as inducible cell lines, we investigated the requirements of apoptosis after treatment with selective Aurora-A and Aurora-B inhibitors in human colon carcinoma cells. From our results, we suggest the following model describing the induction of apoptosis after treatment with these drugs. The abrogation of normal chromosome alignment by MLN8054 treatment leads to a transient mitotic arrest that is not maintained, possibly due to the ability of MLN8054 to override an activated spindle checkpoint. Subsequently, MLN8054-treated cells exit from mitosis and activate a p53- and p21-dependent postmitotic G_1 checkpoint that is required not only for G_1 arrest but also for the induction of apoptosis. In addition, the p53-dependent *BAX* induction is also required for the execution of apoptosis. Thus, important determinants for the efficacy of MLN8054 are an unscheduled exit from mitosis, an intact postmitotic G_1 checkpoint, and a functional Bax-dependent apoptotic pathway in human colon carcinoma cells.

In comparison, ZM447439 treatment causes severe chromosome alignment defects, which do not result in a mitotic arrest, probably due to the abrogation of the spindle checkpoint by ZM447439. Similar to MLN8054 treatment, inhibition of Aurora-B causes a strong activation of the postmitotic G_1 checkpoint, which does, surprisingly, not result in arrest in G_1. ZM447439-treated cells endoreduplicate, and this is required for the subsequent induction of apoptosis, which is dependent on basal levels of Bax. Thus, efficacy of ZM447439 in human colon carcinoma cells requires an aberrant exit from mitosis in the presence of misaligned chromosomes but not an intact spindle checkpoint, nor a functional postmitotic G_1 checkpoint, nor an accumulation of Bax.

Our data indicate that overriding the spindle checkpoint by both Aurora inhibitors is required for their efficacy. Consequently, the induction of apoptosis on Aurora kinase inhibition was found to be independent of the spindle checkpoint in human colon carcinoma cells and might rather depend on the induction of severe aneuploidy as suggested before (25) than on inducing a prolonged mitotic arrest as observed after treatment with chemotherapeutic spindle-damaging drugs such as Taxol or various *Vinca* alkaloids (1). It has been shown previously that inhibition of Aurora-B leads to silencing of the mitotic checkpoint (9, 10), although it is still unclear whether Aurora-B has a direct function in spindle checkpoint signaling or whether checkpoint silencing is just the consequence of the function of Aurora-B to resolve faulty kinetochore attachments (12). On the other hand, it was surprising to find that the selective inhibition of Aurora-A by MLN8054 also causes an override of the spindle checkpoint, a result that has been also shown most recently by Wysong and colleagues (37). At present, it is unclear whether Aurora-A has a direct role in regulating the spindle checkpoint and it is not clear whether a weak inhibition of Aurora-B by MLN8054 might contribute to the checkpoint override. We found that treatment with both ZM447439 and MLN8054 led to diminished amounts of Bub1 and BubR1 at kinetochores, suggesting that both Aurora-A and Aurora-B either might contribute to spindle checkpoint function or might be required to activate the checkpoint indirectly by generating unattached kinetochores. Clearly, more work is required to further investigate the roles of the Aurora kinases in the spindle checkpoint.

Nevertheless, because the spindle checkpoint is overridden by Aurora inhibitors, it is conceivable that the induction of apoptosis occurs independent of a functional spindle checkpoint. This is in contrast to spindle-damaging drugs including Taxol or *Vinca* alkaloids that are frequently used in the clinic and whose efficacy depends on a functional spindle checkpoint (22, 27, 28). Thus, based on our results obtained in colon carcinoma cells, we expect that inhibition of Aurora-A or Aurora-B might be efficient even in tumors harboring an impaired spindle checkpoint. It will be interesting to see whether this holds true in other human tumor entities.

A general feature of the mechanism of antimitotic drugs seems to be the activation of a p53-dependent postmitotic G_1 checkpoint (32, 38). On prolonged treatment with spindle damaging agents, cells first activate the spindle checkpoint and delay in mitosis, which is followed by an unscheduled exit from mitosis leading to the activation of the postmitotic checkpoint, which arrests cells in a p53- and p21-dependent manner in G_1. Failure of this checkpoint (e.g., on inactivation of p53) results in endoreduplication. Thus, the postmitotic G_1 checkpoint might act as a second fail-safe mechanism following a faulty mitosis (32, 38). We showed that inhibition of either Aurora-A or Aurora-B activates the postmitotic checkpoint. Surprisingly, the checkpoint activation causes a G_1 arrest only after MLN8054, which is required to induce apoptosis, but not after ZM447439 treatment, whose efficacy even requires a severe polyploidization. Interestingly, the latter might be related to the most recently discovered phosphorylation of the Rb tumor suppressor protein by Aurora-B, which might contribute to the cell cycle arrest in the postmitotic G_1 phase on unscheduled exit from mitosis (39). In agreement with this, we found that endoreduplication, and thus apoptosis, after Aurora-B inhibition by ZM447439 is not dependent on p53. Thus, based on these results in colon carcinoma cells, Aurora-B inhibitors are expected to be efficient in *TP53*-deficient tumors. In fact, this was seen in

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TP53-deficient PC-3 prostate cancer xenografts as well as in human lung and esophageal adenocarcinoma cell lines after treatment with high concentrations of MLN8054 compatible with Aurora-B inhibition (17, 40). Similarly, apoptosis is readily induced in *TP53*-deficient cancer cell lines derived from different tumor entities after treatment with VX-680 (41).

In contrast, selective inhibition of Aurora-A (at MLN8054 concentrations of 0.5 μmol/L) causes a postmitotic G_1 arrest, which is required for the induction of apoptosis in our colon carcinoma cell system. Thus, based on this, selective Aurora-A inhibitors might be more efficient in *TP53*-proficient tumor cells. In fact, low concentrations of MLN8054 that mediate selective inhibition of Aurora-A induced apoptosis more efficiently in *TP53*-proficient HCT116 cells than in *TP53*-deficient PC-3 cells (17).

In our work presented here, we focused on pathways that are activated in response to Aurora kinase inhibition. Thus, we define a functional spindle checkpoint, the p53-dependent postmitotic G_1 checkpoint, and Bax as critical regulators of apoptosis after Aurora kinase inhibition in colon carcinoma cells. Additional molecular parameters are also critical for the efficacy of Aurora kinase inhibitors. For instance, for the Aurora-B inhibitor AZD1152, the avidin-biotin complex method transporters multidrug resistance 1 and breast cancer resistance protein were found to mediate resistance, indicating that this compound is a substrate for efflux pumps (42).

In addition, the status of the target kinases itself as well as proteins regulating the function of the Aurora kinases might also represent important determinants for the efficacy of Aurora kinase inhibitors. In fact, it has been shown that forced expression of Aurora-A decreases and its down-regulation increases the sensitivity toward Aurora inhibitors in multiple myeloma (36). Remarkably, a high expression of the hyaluronan-mediated motility receptor (HMMR, RHAMM), which occurs frequently in multiple myeloma (43) and in breast cancer (44), has been correlated with increased sensitivity for Aurora inhibitors (36). Interestingly, like Aurora-A, HMMR/RHAMM localizes to centrosomes where it regulates the assembly of the mitotic spindle. Moreover, HMMR/RHAMM interacts with TPX2, an important coregulator of Aurora-A (45). Thus, overexpression of HMMR/RHAMM in cancer cells might negatively interfere with Aurora-A activity, leading to a greater sensitivity toward Aurora kinase inhibitors.

Furthermore, it has been shown that mutations within the *Aurora-B* gene are generated on drug treatment and this can lead to resistance toward Aurora-B kinase inhibitors, including ZM447439 (46).

Together, it should be taken into account that multiple parameters can mediate resistance toward Aurora kinase inhibitors. Clearly, much more work on the mechanisms of action of Aurora kinase inhibitors in various human tumor entities is needed to fully understand the different routes of resistance. The ultimate goal is a tailored treatment using various chemotherapeutic drugs or even a combination of different drugs depending on the genetic predisposition of the tumor to circumvent the mechanisms of resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

Determinants for the efficiency of anticancer drugs targeting either Aurora-A or Aurora-B kinases in human colon carcinoma cells

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