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Aurora kinase inhibitor ZM447439 induces apoptosis via mitochondrial pathways

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ABSTRACT

ZM447439 (ZM) is a potent and selective inhibitor of aurora-A and -B kinase with putative anti-tumoral activity. Inhibitors of aurora kinases were shown to induce apoptosis *in vitro* and *in vivo*. To investigate the underlying mechanisms, cell death pathways triggered by ZM was analysed in HCT-116 colorectal cancer cells. Through correlation of polyploidization and apoptosis in different knockout cells, the interrelation of these cellular responses to ZM was investigated. ZM induced apoptosis in a concentration- and time-dependent manner. ZM-induced apoptosis was associated with an upregulation of p53, breakdown of the mitochondrial membrane potential ($\Delta\Psi$m) and activation of caspase-3. To precisely define key components for ZM-induced apoptosis, knockout cells lacking p53, Bak, Bax or both Bak and Bax were used. Lack of p53 reduced ZM-induced apoptosis and breakdown of $\Delta\Psi$m, while lack of Bak, Bax or both almost completely inhibited apoptosis and breakdown of $\Delta\Psi$m. Since no difference in apoptosis induction was detectable between HCT-116 cells lacking Bak, Bax or both, apoptosis induction depended non-redundantly on both Bak and Bax. Phenomenally, ZM induced notable polyploidization in all examined cells, especially in p53−/− cells. A correlation between polyploidization and apoptosis was observed in wild-type, and also in p53−/− cells, albeit with a modest extent of apoptosis. Moreover, in Bak−/−, Bax−/− and Bak/Bax−/− cells apoptosis was totally inhibited in spite of the strongest polyploidization, suggesting apoptosis may be a secondary event following polyploidization in HCT-116 cells. Thus ZM-induced apoptosis depends not only on polyploidization, but also on the intracellular apoptotic signaling.

1. Introduction

The aurora kinases comprise a family of serine/threonine kinases that are essential for coordinated mitotic progression [1]. To date, three members of the aurora kinase family have been identified, namely aurora-A, -B, and -C kinases. Aurora-A was shown to be essential for the assembly of the spindle apparatus and accurate chromosome segregation [2]. Aurora-B is a chromosome passenger protein kinase, required for phosphorylation of histone H3, chromosome segregation, and cytokinesis [3]. Aurora-C has also been shown to be a chromosome passenger protein that may replace the function of aurora-B and is required for cytokinesis [4].

Aurora kinases are strongly associated with human cancer [5–7]. Overexpression of aurora-A kinase leads to aberrant mitosis and transforms mammary epithelial and embryonic fibroblast cells to genetically unstable aneuploid cells with multiple centrosomes [8]. Aurora-B kinase is also expressed at high levels in tumor cells [3,6]. Overexpression of aurora-B kinase correlates with the clinical Dukes' stage in primary colon cancers and is closely implicated in tumor progression [6]. Furthermore overexpression of aurora kinases is found in a wide array of human cancers including colorectal-, breast-, gastric-, ovarian-, and pancreatic cancer [3,5,9,10].

Several inhibitors of aurora kinases including Hesperadin, ZM447439 (ZM), VX680 and AZD1152 have been developed as anti-cancer agents with encouraging anti-tumoral potential *in vitro* and *in vivo* [7,11,12]. However the underlying mechanisms of the anti-neoplastic activities of these drugs are still poorly understood.

Recent studies suggest that apoptosis induction may be involved in mediating anti-tumoral effects of aurora kinase

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inhibitors [11–15]. In this regard, it has been shown that inhibition of aurora-A kinase causes accumulation of p53 via reversal of the aurora-A kinase triggered destabilization and proteolysis of p53 through Mdm2 [14]. Thus, inhibition of aurora-A might increase the sensitivity to undergo apoptosis [14]. More importantly, till now it was hypothesized that aurora kinase inhibitor-induced apoptosis depends on polyploidization, the primary phenomenal change induced by all aurora kinase inhibitors [11,16–18]. The chromosomal chaos may cause cellular stress and lead to apoptosis [11,13,16–20]. Since the integrity of the p53-p21$^{Waf1/Cip1}$ pathway plays an important role in the post-mitotic checkpoint regulation, aurora kinase inhibitors induce more polyploidization in cell systems with abrogated p53, subsequently followed by more apoptosis [13,16]. However, other studies have demonstrated that aurora kinase inhibitor-induced DNA damage upregulates p53 and induces apoptosis in a p53-mediated manner [18,21]. Thus, the exact mechanisms of aurora kinase inhibitor-induced apoptosis seem to be complicated and need to be further investigated.

Therefore, our study was designed to determine the cellular signaling pathways of ZM-induced apoptosis and polyploidization in HCT-116 colorectal tumor cells, using wild-type and cells lacking p53, Bak, Bax or both Bak and Bax. ZM-induced apoptosis was totally inhibited in cells lacking Bak, Bax or both, suggesting ZM induces apoptosis via mitochondrial pathways. In cells lacking p53, ZM-induced apoptosis was significantly attenuated, while polyploidization increased, compared to wild-type respectively, indicating the pro-apoptotic role of p53 in HCT-116 cells.

2. Materials and methods

2.1. Chemicals and drugs

ZM447439 is synthesized by AstraZeneca Pharmaceuticals [16]. ZM was dissolved in sterile DMSO to 10 mM (stock solution) and stored at −20 °C. Tetramethyl-rhodamine-ethylester-perchlorate (TMRE) was from Molecular Probes (Mobitec, Goettingen, Germany). Hoechst33342 was purchased from Calbiochem (Bad Soden, Germany) and dissolved in distilled water as a 1.5 mM stock solution.

The rabbit anti-p53, rabbit anti-Bak, rabbit anti-Bax, rabbit anti-caspase-3, rabbit anti-cleaved caspase-3 and rabbit anti-GAPDH (14C10) were from Cell Signaling (New England Biolabs, Schwalbach, Germany) and the horseradish peroxidase-conjugated sheep anti-mouse IgG was from Amersham-Pharmacia Biotech (Freiburg, Germany). Caspase-8 was detected with a mouse monoclonal antibody directed against the p18 subunit as described previously [26] used as a 1:10 dilution of the hybridoma supernatant. All other chemicals were purchased from Sigma–Aldrich (Deisenhofen, Germany) if not otherwise indicated.

2.2. Cell culture

HCT-116 colorectal tumor cells were purchased from ATCC (Bethesda, MD, USA). HCT-116$^{p53-/-}$, HCT-116$^{Bak-/-}$, HCT-116$^{Bax-/-}$, HCT-116$^{Bak/Bax-/-}$ are created in Vogelstein's laboratory and provided as generous gift from P. Daniel [45]. Cells were grown in RPMI 1640 medium (Gibco Life Technologies, Eggenstein, Germany) with 10% fetal calf serum and maintained in a humidified incubator at 37 °C with 5% CO_2.

2.3. Apoptosis counting

Cells were stained with Hoechst33342 (final concentration 1.5 mM) and propidium iodide (PI, final concentration 50 μg/ml) for 15 min. Cell morphology was determined by fluorescence microscopy (Zeiss Axiovert 200, Carl Zeiss, Jena, Germany) using an excitation wavelength filter of 380 nm. Cells were analysed with ×40 magnification and documented using a CCD camera device (Zeiss Axiocam MRm). For apoptosis counting three view fields were randomly selected for each group under microsope and 100 cells were counted in each field. The mean of apoptotic cells was calculated. The results of triple experiments were presented with SD.

2.4. Determination of mitochondrial transmembrane potential in flow cytometry

The mitochondrial transmembrane potential ($\Delta\Psi$m) was analysed using the $\Delta\Psi$m-specific stain TMRE (Molecular Probes, Mobitech, Goettingen, Germany). Briefly, 2×10^5 cells were loaded for 10 min at 37 °C with 25 nm TMRE and subsequently analysed by flow cytometry. Pre-incubation with 1 μm of the proton ionophor carbonylcyanide-m-chlorophenylhydrazone (CCCP, Sigma–Aldrich, Deisenhofen, Germany) was used as a positive control for complete depolarization of the mitochondrial membrane potential. Data were collected using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). For each sample, 10,000 events were collected. Data were analysed with FCS Express (De Novo Software, Los Angeles, CA).

2.5. Determination of polyploidy and sub-diploid population in flow cytometry

The sub-diploid (sub-G1) population and polyploidization were analysed in flow cytometry using propidium iodide (Calbiochem, Karlsruhe, Germany) staining, as described before [22]. Briefly, 2×10^5 cells were collected and stained with Nicoletti's stain solution (0.1% natrium citrate, 0.1% Triton X-100, 50 μg/ml PI). After 30 min incubation protected from light, cells were analysed in flow cytometry 200–500 events/s. Data were collected using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). For each sample, 2×10^4 events were collected. Data were analysed with FCS Express.

2.6. Statistical evaluation

Experiments were at least performed in triplicates. Results are means ± standard deviation (SD) except indicated otherwise. $p < 0.05$ was considered as significant.

3. Results

3.1. Aurora kinase inhibitor ZM447439 induced apoptosis in HCT-116 wild-type cells in a dose- and time-dependent manner

To verify the inhibitory potential of ZM to aurora kinases, phosphorylation of histone H3 (Ser 10), a main substrate of aurora kinases, was determined by Western-blotting. ZM inhibited the phosphorylation of histone H3 (Ser 10) in a concentration-dependent manner (Fig. 1a), with 5 μM ZM being sufficient to almost totally inhibit the kinase activity of aurora kinases 24 h after the exposure.

Treatment with aurora kinase inhibitor (ZM447439) induced significant apoptosis in HCT-116^{wt} colorectal tumor cells. Typical morphological changes of apoptosis, such as DNA condensation and fragmentation were observed using Hoechst/PI double staining (Fig. 1b). To quantify the ZM-induced apoptosis, apoptotic cells were counted 24 h, 48 h and 72 h after incubation with different concentrations of ZM (0 μM, 0.5 μM, 5 μM). ZM induced apoptosis in a concentration- and time-dependent manner (Fig. 1c, 24 h, 48 h, 72 h after the treatment of 5 μM ZM, 14.3%, 20%, 32.8% apoptotic cells; and 72 h after the administration of 0.5 μM, 2 μM,

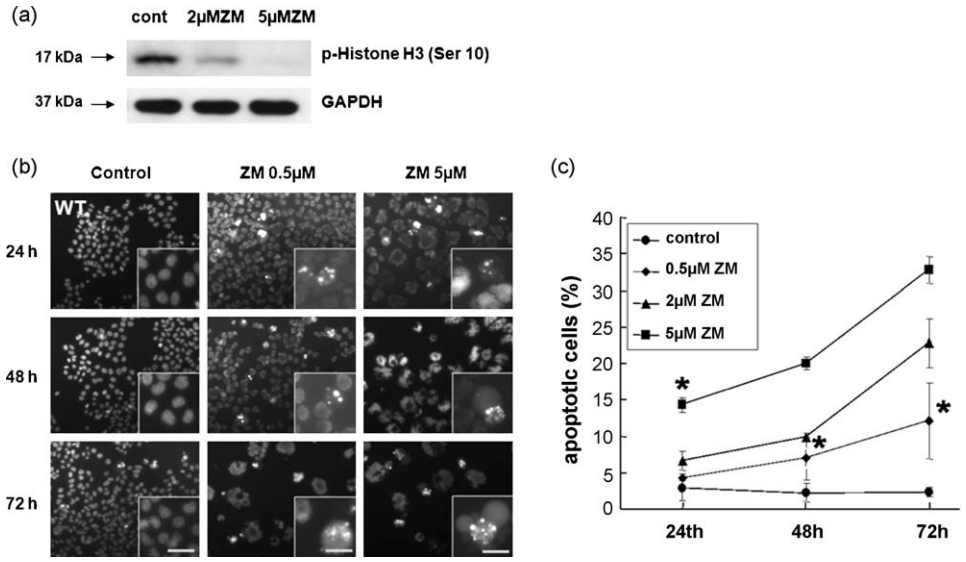

Fig. 1. (a) Western-blotting of phosphorylated histone H3 (Ser. 10). The phosphorylation of histone H3 was inhibited by ZM in a concentration-dependent manner, verifying its inhibitory potential to aurora-B kinase. GAPDH was used as loading control. (b) HCT-116 wild-type cells were treated with ZM447439 (ZM) with indicated concentrations and stained with Hoechst and PI. Typical apoptotic changes were shown 24 h, 48 h and 72 h after incubation. Representative photos were shown here. Bar, 50 μm. (c) HCT-116 wild-type cells were treated as described before. Apoptotic cells were counted under fluorescent microscope in three independent view fields in every group. Treatment with ZM induced notable apoptosis in HCT-116 wild-type tumor cells in a concentration- and time-dependent manner. Data show means ± SD ($n = 3$). Asterisks indicate a significance of $p < 0.05$, compared to control groups, respectively.

5 μM ZM, 12.2%, 22.8%, 32.8% apoptotic cells *vs.* 2.3% in control group, $p < 0.01$). The strongest induction of apoptosis was observed 72 h after the administration of 5 μM ZM (32.8% *vs.* 2.6% in control group, $p < 0.01$).

Inhibition of aurora kinase interferes with spindle checkpoints and disturbs mitotic progression. This finally leads to endoreduplication and polyploidization, followed by apoptosis [14,16]. As expected, we observed a concentration- and time-dependent increase in the frequency of "giant cells" after exposure to ZM (Fig. 1b).

3.2. ZM447439 induced a breakdown of mitochondrial membrane potential and an increase of sub-diploid population in HCT-116 wild-type cells

In parallel to the morphological analysis, quantification of the sub-diploid (sub-G1) population after PI staining was employed to determine the extent of apoptosis induction by ZM. A marked increase in sub-G1 cells was visible after treatment with 5 μM ZM (Fig. 2a, 24 h, 48 h, 72 h after treatment of 5 μM ZM, 16.5%, 23.5%, 29% *vs.* 8.2%, 5.3%, 4.8% in control groups, respectively, $p < 0.05$).

Many anti-neoplastic drugs induce apoptosis of cancer cells via mitochondrial apoptotic pathways [23–26]. A hallmark of death induction via these pathways is a rapid and early breakdown of mitochondrial membrane potential. Thus, in the next step we analysed the integrity of mitochondrial function after treatment with ZM. ZM significantly induced breakdown of the mitochondrial membrane potential ($\Delta\Psi m$) in a time- and concentration- (data not shown) dependent manner, as determined by FACS analysis of cells stained with potential sensitive dye tetramethyl-rhodamine-ethylester-perchlorate (TMRE) (Fig. 2b, 24 h, 48 h, 72 h after treatment of 5 μM ZM, 18.2%, 43.3%, 59.5% *vs.* 8.4%, 8%, 10% in control groups, respectively, $p < 0.05$).

3.3. ZM447439 induced cleavage of caspase-3 but not caspase-8 in HCT-116 wild-type cells

To further verify ZM-induced apoptotic cell death on protein level, we examined the effect of ZM on activation of caspase-3 using Western-blotting. Treatment of HCT-116 wild-type cells with ZM for 48 h caused a clear increase of 17 and 19 KD cleaved

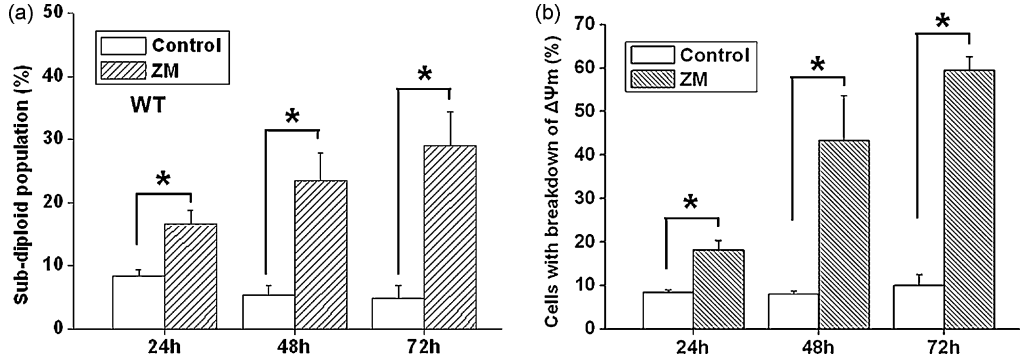

Fig. 2. (a) FACS analysis of ZM447439-treated HCT-116 wild-type cells using PI staining. ZM447439 significantly increased apoptotic sub-G1 population in a concentration- (data not shown) and time-dependent manner. (b) FACS analysis of ZM447439-treated HCT-116 wild-type cells using potential specific dye TMRE staining. ZM also induced breakdown of mitochondrial membrane potential in a concentration- (data not shown) and time-dependent manner. Asterisks indicate $p < 0.05$.

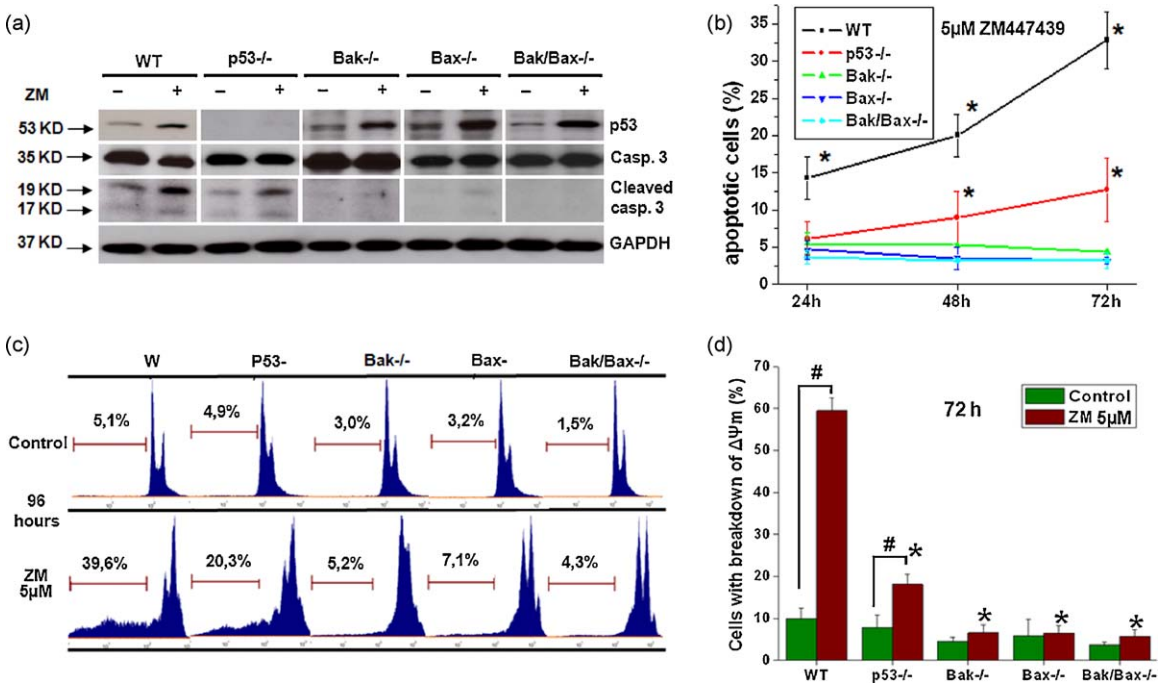

Fig. 3. (a) Western-blotting of p53, caspase-3, cleaved caspase-3 in HCT-116 WT, p53−/−, Bak−/−, Bax−/− and Bak/Bax−/− cells after ZM treatment. ZM447439 upregulated p53 in WT, Bak−/−, Bax−/− and Bak/Bax−/− cells, while p53−/− was verified. ZM447439 induced a distinct cleavage of pro-caspase-3 in WT cells and an attenuated cleavage in p53−/− cells, but not in Bak−/−, Bax−/− and Bak/Bax−/− cells. GAPDH was used here as loading control. (b) Apoptosis counting was undertaken in HCT-116 WT, p53−/−, Bak−/−, Bax−/−, Bak/Bax−/− cells after ZM treatment. Mean of triple experiments was shown here with standard error. Asterisk $p < 0.05$. (c) Flow cytometry analysis of WT, p53−/−, Bak−/−, Bax−/−, Bak/Bax−/− cells after 96 h incubation of 5 μM ZM using PI staining. Representative histograms were shown here. (d) Flow cytometry analysis of mitochondrial membrane potential ($\Delta\Psi$m) in WT, p53−/−, Bak−/−, Bax−/−, Bak/Bax−/− cells after 72 h incubation of 5 μM ZM using TMRE staining. Knockout of p53 significantly reduced ZM-induced breakdown of $\Delta\Psi$m ($p < 0.01$), while knockout of Bak, Bax or both abolished ZM-induced breakdown of $\Delta\Psi$m ($p < 0.01$).

fragment of caspase-3 (the first 2 lanes in Fig. 3b), indicating the activation of caspase-3.

Caspase-8 is a classical executor protein in the extrinsic apoptotic pathway. Although activation of caspase-8 is not uniquely associated with cell death induction via death receptors, a rapid and early activation of caspase-8 may be an indicator for an involvement of this death cascade. Therefore Western-blots with activation-specific antibody directed against caspase-8 were performed. ZM did not induce cleavage of caspase-8 in apoptotic HCT-116 cells, suggesting that the extrinsic pathway is not involved in the ZM-induced apoptosis (Supplementary Fig. 1a).

3.4. zVAD potently inhibited the ZM447439-induced apoptosis

Since other pro-apoptotic factors, such as apoptosis-inducing factor, Endo-G, may also contribute to apoptosis induction [27,28], we attempted to confirm caspase dependence by pre-incubation with the pan-caspase inhibitor zVAD. The drug was administrated to HCT-116 wild-type cells 10 min prior to 5 μM ZM treatment, and apoptosis induction was analysed using Hoechst/PI double staining. Treatment with zVAD totally inhibited ZM-induced apoptosis in HCT-116 wild-type cells at all examined time points (24 h, 48 h, 72 h after the treatment of zVAD and ZM, 1.5%, 1.7%, 2.7% *vs.* 13.7%, 20.7%, 33.3% in the ZM groups, respectively, Fig. 4a and b), indicating a complete caspase-dependency in the ZM-induced apoptosis. In the presence of zVAD, the fraction of unspecific cell death induced by ZM (e.g. necrosis) increased, compared to control group (data not shown).

3.5. P53 acted as a pro-apoptotic factor in the ZM447439-induced apoptotic pathways

To evaluate the role of p53 during ZM-induced apoptosis, we firstly determined the level of p53 by Western-blotting. After 48 h, ZM upregulated p53 in HCT-116 wild-type cells (Fig. 3a). With the observation that p53 is upregulated after treatment with ZM a potential prerequisite for p53-mediated apoptosis is fulfilled. However, to functionally validate the involvement of p53, HCT-116$^{p53-/-}$ cells were employed. Knockout of p53 was verified in Western-blotting (Fig. 3a).

Knockout of p53 significantly reduced ZM-induced apoptosis, both in the apoptotic cell counting (Fig. 3b, $p < 0.01$) and in FACS assays presented as sub-G1 population (Fig. 3c, 20.3% *vs.* 39.6% in wild-type cells). Knockout of p53 also significantly protected cells from ZM-induced breakdown of mitochondrial membrane potential (Fig. 3d, 10.1% *vs.* 59.5% in wild-type cells 72 h after the treatment, $p < 0.01$). Consistently, on the protein level knockout of p53 reduced the cleavage of caspase-3 (Fig. 3a).

3.6. ZM447439-induced apoptosis depended on expression of both Bak and Bax

As shown above, treatment of cells with ZM impaired mitochondrial integrity indicating that ZM induces apoptosis via mitochondrial apoptotic pathways. Since Bak and Bax are the two most essential pro-apoptotic proteins for mitochondrial apoptotic events [29], HCT-116$^{Bak-/-}$, HCT-116$^{Bax-/-}$ and HCT-116$^{Bak/Bax-/-}$ cells were used to analyse the roles of Bax and Bak for ZM-induced cell death. Knockout of Bak, Bax or both was verified in Western-blotting (Supplementary Fig. 1b).

Knockout of Bak or/and Bax almost totally abrogated ZM-induced apoptosis as detected by apoptosis counting (Fig. 3b). In FACS assay with PI staining, the ZM-induced increase of sub-diploid population was also significantly reduced in the absence of Bak, Bax or both (2.2%, 3.9%, 2.8% *vs.* 34.4% in wild-type, normalized to control groups respectively, Fig. 3c). Consistently, knockout of Bak, Bax or both inhibited the ZM-induced breakdown of $\Delta\Psi$m (Fig. 3d, $p < 0.01$). There was no significant difference between Bak−/−, Bax−/− and

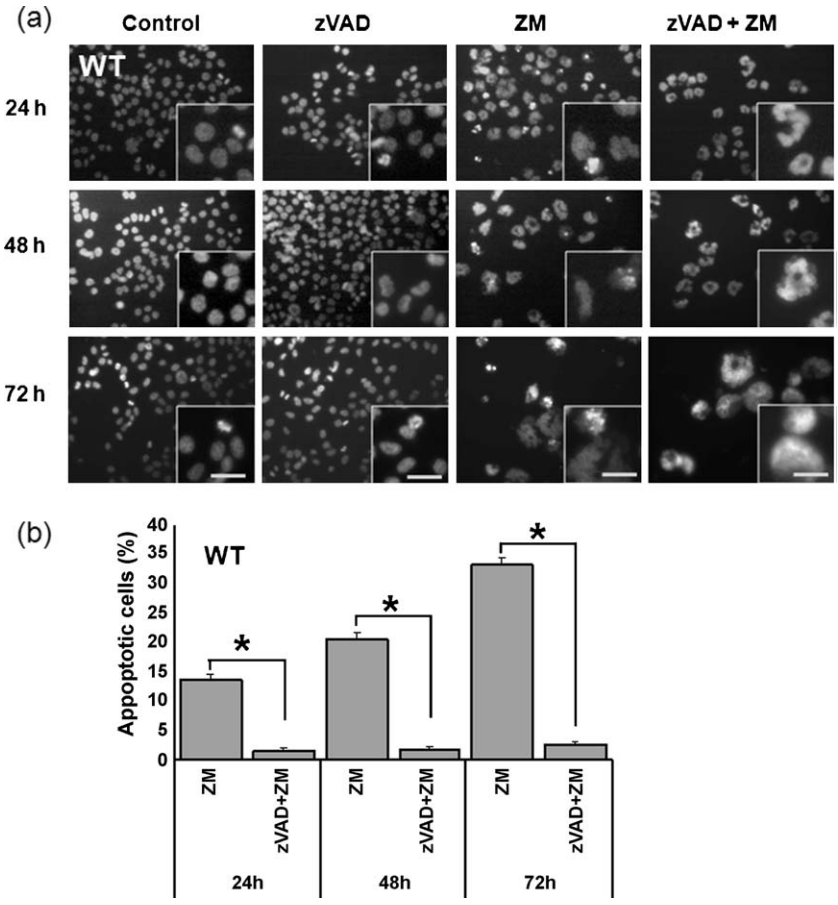

Fig. 4. A pan-caspase inhibitor zVAD inhibited ZM-induced apoptosis. (a) At all examined time points (24 h, 48 h and 72 h), 20 nM zVAD blocked 5 μM ZM-induced apoptosis. (b) The quantitative analysis of ZM-induced apoptosis in the presence of zVAD. 20 nM zVAD totally blocked ZM-induced apoptosis at all examined time points ($p < 0.01$). Triple experiments were performed. Data show means ± SD ($n = 3$). Bar, 50 μm.

Bak/Bax−/− cells. Moreover, we determined the level of p53 and the cleavage of caspase-3 in HCT-116$^{Bak-/-}$, HCT-116$^{Bax-/-}$ and HCT-116$^{Bak/Bax-/-}$ cells using Western-blotting. Although treatment of ZM upregulated p53 in these 3 knockout cell lines, the activation of caspase-3 was totally inhibited (Fig. 3a). Similar to wild-type cells, ZM did not induce the cleavage of caspase-8 in any tested cells (Supplementary Fig. 1a).

3.7. Knockout of P53 accelerated the ZM447439-induced polyploidization

Since aurora kinases play key roles in the mitotic progression, inhibition of aurora kinase primarily disturbs mitotic progression and interferes with spindle checkpoints, leading to polyploidization subsequently [13,16]. Consistently, we observed ZM-induced "giant cells" with noticeable polyploidy in all examined cell lines after Hoechst staining (Fig. 5). The polyploidization was more pronounced in the absence of p53 than in the presence of p53, as observed under microscope after Hoechst staining (Fig. 5) and determined using flow cytometry (Fig. 5, 63% *vs.* 15%, 23%, 32%, 19% in WT, Bak−/−, Bax−/−, Bak/Bax−/− cells >8N DNA content, 48 h after 5 μM ZM treatment).

3.8. Interrelation between polyploidization and apoptosis

Several studies have suggested a correlation between polyploidization and apoptosis mediated by aurora kinase inhibitors [13,16]. Moreover, accelerating polyploidization through compromising the post-mitotic checkpoint induced an increase of apoptosis [13,16]. To further gain an insight into the interrelation between polyploidization and apoptosis, we tried to correlate these two cellular responses to ZM. In HCT-116 wild-type cells, the ZM-induced increase of polyploidization correlated with the increase of apoptosis during the incubation time (Fig. 6a, 24 h, 48 h, 72 h after 5 μM ZM treatment). In p53−/− cells this correlation still existed albeit with a moderate extent of apoptosis, although the polyploidization was accelerated. In cells with disrupted intrinsic apoptotic pathways through knockout of Bax, Bak or both, no correlation was observed, since ZM-induced apoptosis was totally inhibited (only Bax−/− was shown here. Bak−/− and Bak/Bax−/− cells were almost identical to Bax−/− cells concerning cellular response to ZM. Supplementary Fig. 2.)

The interrelation was also demonstrated by overlaying FACS histograms using PI staining. Again, p53−/− cells were more prone to polyploidization after ZM treatment, compared to p53 wild-type cells (WT and Bax−/−, 48 h after treatment of ZM). The apoptotic sub-G1 population was reduced in p53−/− cells and almost totally inhibited in Bax−/− cells, compared to wild-type cells respectively (Fig. 6a and b). Interestingly, after prolonging the incubation time up to 96 h, Bax−/− cells showed even more polyploidization than p53−/− cells (Fig. 6b).

4. Discussion

Aurora kinase inhibitors are a new class of substances with putative anti-neoplastic activity. In the present study, we analysed the molecular mechanisms of cell death induction triggered by aurora kinase inhibitor, ZM447439 [16]. Our data suggest that ZM-

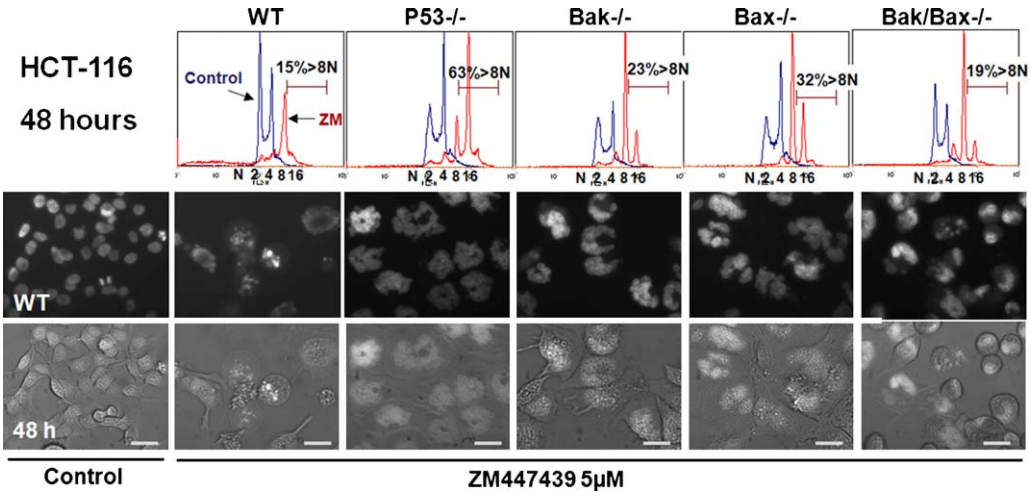

Fig. 5. Polyploidy determination in FACS with corresponding photos under fluorescent microscope using PI staining. After 48 h incubation with 5 μM ZM, cells were collected and analysed in FACS using PI staining. ZM induced polyploidization more efficiently in p53−/− cells than cells with intact p53. The corresponding photos of the treated cells were also shown. Untreated wild-type cells were used here as negative control. Bar, 50 μm.

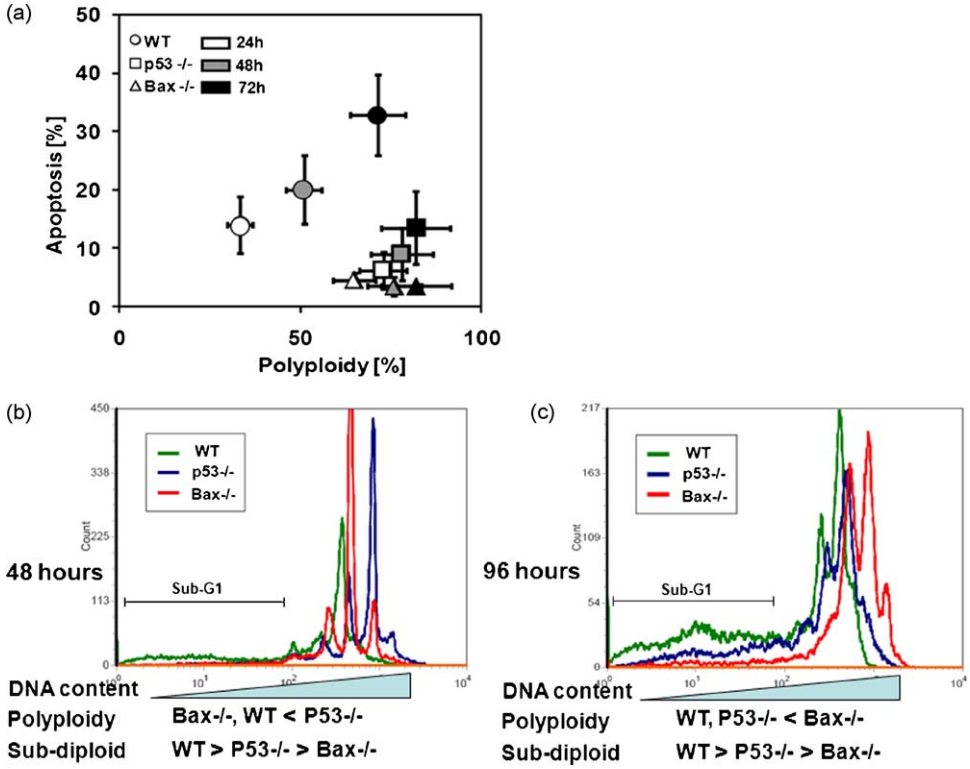

Fig. 6. Interrelation between ZM447439-induced polyploidization and apoptosis. (a) In HCT-116 wild-type cells (WT), ZM-induced apoptosis was correlated to polyploidization measured in FACS analysis using PI staining, as incubation time prolonged from 24 h to 72 h. Polyploidization was defined here as cells more than 4N DNA content. In p53 knockout cells, ZM-induced apoptosis still correlated to polyploidization, but in a modest extent of apoptosis. On the contrary, knockout of Bak, Bax or both totally blocked ZM-induced apoptosis, although polyploidization continued during the incubation time. (b) Direct comparison of ZM-induced polyploidization and apoptosis in HCT-116 WT, p53−/− and Bax−/− cell lines by overlaying FACS histograms after PI staining. Results of 48 h and 96 h ZM treatment were shown here.

induced apoptosis was mediated strictly via Bak/Bax dependent mitochondrial apoptotic pathways. Apoptosis was not detectable in either Bax or Bak knockout cells indicating a non-redundant dependency on Bak and Bax.

Bak and Bax are the two most important pro-apoptotic proteins modulators of the intrinsic apoptotic pathways that can directly perturb mitochondrial membrane potential [30]. However it is still unclear how exactly activation of Bak and Bax leads to mitochondrial outer membrane permeabilization (MOMP), the point-of-no-return in apoptosis induction. Some experiments indicated Bak or Bax might act independently in apoptosis induction [31,32]. However, recent studies revealed that Bak and Bax might act functionally in a non-redundant fashion during infection- and cisplatin-induced apoptosis [33,34]. In accordance with these findings, we demonstrated that ZM-induced apoptosis requires both Bak and Bax (Fig. 7), since knockout of Bak, Bax or both caused total resistance to apoptosis. Thus similar to cisplatin-induced apoptosis, ZM-induced apoptosis seems to rely on a complex interplay of both Bax and Bak.

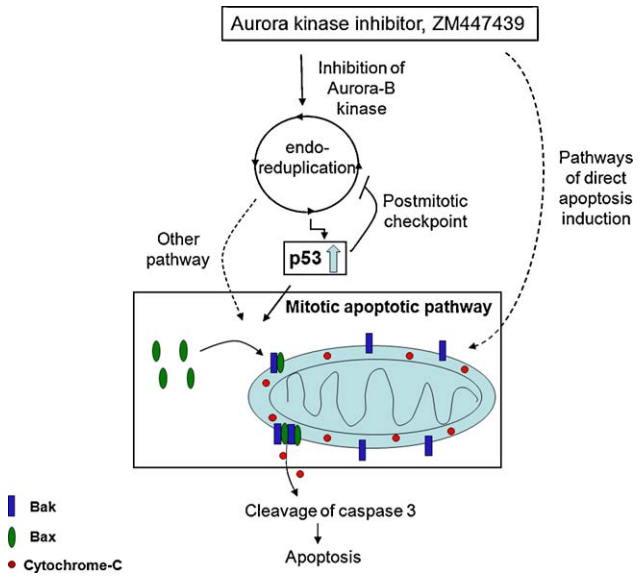

Fig. 7. Hypothetic schematic diagram of the apoptotic signaling induced by ZM447439. Clear signaling cascades are shown in solid lines and hypothetical parts in dotted lines.

In parallel to intrinsic apoptosis cascades, the extrinsic apoptosis cascade is the other important pathway to induce apoptosis. Paradigmatically, apoptosis-inducing ligands (e.g. CD95, tumor necrosis factor) initiate apoptosis via activation of their receptors [35,36], followed by proteolytic activation of caspase-8 [37]. A recent study in glioma cells showed that inhibition of aurora-B kinase upregulated tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor, and enhanced TRAIL-mediated apoptosis [38]. However in the present study, the strict dependency on Bak and Bax and the absence of activation of caspase-8 suggested that the extrinsic apoptotic pathway was not operative in HCT-116 cells.

An easy interpretation of cellular responses to ZM44739 is generally hampered by the fact that ZM is a selective inhibitor to both aurora-A and -B kinases [16]. Concerning cell cycle progression, the ZM-induced polyploidization was suggested to depend on inhibition of aurora-B kinase, because inhibition of aurora-B kinase abrogates the spindle checkpoint and thus bypasses the requirement of aurora-A kinase [39]. However, beside its function in spindle checkpoint, aurora-A kinase can downregulate p53 through stimulating its ubiquitination via Mdm2 [12,14]. Thus, inhibition of aurora-A kinase by ZM would stabilize p53 and induce more sensitivity to apoptosis, since the inhibitory potential of ZM to aurora-A kinase (IC50 110 nM) was verified in a previous study [16]. This mechanism may be responsible for a direct apoptosis induction that partially contributes to the ZM-induced apoptosis (Fig. 7). Currently no data are present allowing an exact estimation of ratio of direct apoptosis induction and apoptosis resulting as secondary event after polyploidization.

Polyploidization is the unique primary cellular response to inhibition of aurora-B, that was suggested to be due to endoreduplication [11–13,16]. Consistently, "giant cells" with pronounced polyploidization were observed after ZM treatment in our experiments (Fig. 5). Furthermore, several recent studies have suggested a direct correlation between polyploidization and apoptosis after inhibition of aurora kinases [13,16]. In the present study, this correlation was observed in HCT-116 wild-type cells, but also in p53−/− cells with a moderate extent of apoptosis during the incubation time (Fig. 6a). Contrarily, disrupted intrinsic apoptotic signaling through knockout of Bax, Bak or both, ZM-induced apoptosis was totally inhibited, although notable polyploidization was observed (Figs. 3 and 6). The disassociation between polyploidization and apoptosis suggested that apoptosis induction may be a secondary event, depends not only on polyploidization but also on the apoptotic pathways. However, the intracellular link connecting polyploidization to apoptosis induction is currently poorly understood.

P53, due to its important roles in different pathways, including apoptosis induction, post-mitotic checkpoint and polyploidy checkpoint, was supposed to be a linkage between polyploidization and apoptosis. This assumption is supported by several studies and some results in the present study. Firstly, p53 was upregulated during endoreduplication and polyploidization after exposure to ZM (Fig. 3a) or VX680, another aurora kinase inhibitor [13]. More exactly, upregulation of p53 was shown to be ATM/ATR-dependent and triggered by aurora kinase inhibitor-induced DNA damage [18]. Moreover, boosting upregulation of p53 through antagonizing Mdm2 acts synergistically with MK-0457 (formerly VX680, a pan aurora kinase inhibitor) to induce apoptosis via mitochondrial pathways [21]. Consistently, in the present study knockout of p53 attenuated ZM-induced apoptosis. In keeping with the loss of post-mitotic checkpoint the knockout of p53 increased polyploidization after ZM treatment. But the increased level of polyploidization does not translate into increased level of secondary apoptosis (Figs. 3 and 6).

This finding seems to be cell type specific, since it has also been reported that abrogating p53 resulted in increased apoptosis through accelerating aurora kinase inhibitor-induced polyploidization [13,16]. A possible explanation for these seemingly contradictory finding may be due to the "dual roles" of p53 in apoptosis induction in response to aurora kinase inhibitor. On the one hand, p53 as a key factor in post-mitotic checkpoint restrains cell cycle through regulating downstream proteins (e.g. p21) after abnormal mitosis and attenuates aurora kinase inhibitor-induced polyploidization, and thus apoptosis [13,16]. On the other hand, p53 is also an important pro-apoptotic factor on different levels. Transcriptionally, p53 can upregulate many pro-apoptotic proteins (e.g. PUMA, NOXA, Bax, Bid), which contribute to apoptosis via intrinsic pathways [40,41]. Moreover, extra-nuclear p53 can also function analogous to BH3-only proteins to induce apoptosis in releasing BH3-only proteins through binding to anti-apoptotic proteins and direct activating apoptotic function of Bak or Bax [42,43]. Thus, the observation that apoptosis induction was attenuated in p53 knockout cells in the present study suggests that the pro-apoptotic effect of p53 is dominant in HCT-116 cells. In fact, apoptosis can be thought of as an endpoint of losing balance between pro- and anti-apoptotic factors favoring pro-apoptotic side. After exposure to ZM447439, DNA damage activated ATM and ATR which are responsible for upregulating p53 in these "giant cells", as cells undergo endoreduplication and polyploidization [18]. After reaching a certain threshold, apoptosis is initiated via intrinsic pathways, requiring both Bak and Bax (Fig. 7). Although a significant p53-dependency was detectable, apoptosis induction was not completely abrogated in p53−/− cells, suggesting that parallel to p53-dependent apoptotic pathways alternative signaling should exist, e.g. p73 [19,44].

It is also to note that the tightness of post-mitotic checkpoint and the susceptibility to apoptosis can be very different in different cell lines in nature. In the present study ZM induced more polyploidization in HCT-116 cells than in other cell lines (HeLa, A549 and HME) in another study [16]. However, using the same HCT-116 cell lines the cellular response (e.g. polyploidization) to ZM is in accordance [18].

Taken together, we showed that aurora kinase inhibitor ZM447439 induced apoptosis in a concentration- and time-dependent manner, following polyploidization. Moreover, we

demonstrated for the first time that apoptosis induced by inhibition of aurora kinases occurs via the mitochondrial pathways, depending on both Bak and Bax. Apoptosis as a secondary event in response to aurora kinase inhibitors, depends not only on polyploidization, but also on the intracellular apoptotic signaling of treated cells. Thus, therapeutic options that stimulate apoptosis may act synergistically with aurora kinase inhibitors to potentiate their anti-tumoral effects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.08.011.

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