

The aurora B kinase inhibitor AZD1152 sensitizes cancer cells to fractionated irradiation and induces mitotic catastrophe

Yungan Tao,¹⁻³ Céline Leteur,^{1,3} Julien Calderaro,⁶ Fiona Girdler,⁷ Ping Zhang,¹⁻³ Valérie Frascogna,^{1,3} Mariana Varna,⁶ Paule Opolon,^{3,4} Maria Castedo,^{3,5} Jean Bourhis,¹⁻³ Guido Kroemer^{3,5,*} and Eric Deutsch^{1-3,*}

¹UPRES EA27-10 Laboratoire Radiosensibilité des Tumeurs et Tissus Sains; Villejuif, France; ²University Paris-Sud XI; Kremlin-Bicêtre, France; ³Institut Gustave-Roussy; Villejuif, France; ⁴UMR 8121; CNRS; Villejuif, France; ⁵U848 INSERM; Villejuif, France; ⁶U728 INSERM; Paris, France; ⁷Faculty of Life Sciences; University of Manchester; Manchester, UK

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AZD1152, an Aurora kinase inhibitor with selectivity for Aurora B kinase, can enhance the effect of ionizing radiation (IR). The aim of this study was to evaluate and to mechanistically explore scheduling effects of AZD1152 on tumor responses to IR, in three different settings: neoadjuvant (AZD1152 before IR), adjuvant (IR before AZD1152), or concomitant treatments (AZD1152 plus one single IR dose). A more pronounced tumor growth delay was observed in the neoadjuvant and adjuvant schedules as compared to the concomitant schedule. However, AZD1152 enhanced the efficacy of IR when concomitant IR was fractionated over several days. Histopathological examination revealed that AZD1152 + IR induced polyploidy, multinucleation and micronuclei *in vivo*. Time-lapse videomicroscopy confirmed that cell death induced by AZD1152 + IR was preceded by multinucleation and the formation of micronuclei, which both are hallmarks of mitotic catastrophe. Caspase inhibition or removal of the pro-apoptotic protein Bax did not ameliorate the long-term cell survival of AZD1152-treated cancer cells. In contrast, a chemical inhibitor of CHK1, Chir124, sensitized cancer cells to the lethal effect of AZD1152. Altogether, these data support the contention that AZD1152 mediates radiosensitization *in vivo* by enhancing mitotic catastrophe, which can be used as a biomarker of treatment efficacy.

Introduction

Aurora B kinase is one of the chromosomal passenger proteins that are essential for a number of processes during mitosis.¹ The expression and activity of Aurora B is regulated by the cell cycle: expression peaks at the G₂-M transition and kinase activity is maximal during mitosis. Aurora B kinase localizes to the centromeres in the early stages of mitosis but relocates to microtubuli at the spindle equator at the onset of anaphase.² During cytokinesis, Aurora B accumulates in the spindle midzone and subsequently at the cleavage furrow and at the midbody.³

Aurora B is responsible for the mitotic phosphorylation of histone H3 (at two serine residues, Ser10 and Ser28) and of CENP-A (at Ser7) that are necessary for chromosome condensation. Aurora B activation is triggered by autophosphorylation after association with its substrate INCENP. In mammalian cells, Aurora B forms a large chromosome passenger complex with INCENP, survivin and borealin,⁴⁻⁷ and is required for the spindle checkpoint.⁸ Aurora B can phosphorylate the microtubule motor protein MCAK (mitotic centromere-associated kinesin),⁹ thereby inactivating its microtubule depolymerase activity and targeting it to the kinetochores where MCAK modifies incorrect kinetochore attachments to the spindle microtubules. Loss

of Aurora B function causes the failure to retain checkpoint proteins at the kinetochore and hence abolishes the taxol-sensitive, tension-sensing spindle checkpoint.^{10,11} Aurora B phosphorylates numerous other proteins that are essential for cytokinesis, such as MKLP-1 (essential for establishment of the central spindle during anaphase), vimentin (an intermediate filament protein participating in formation of the cleavage furrow), and MgcRacGAP (a GTPase-activating protein [GAP] required for cytokinesis).¹²

Aurora B is overexpressed in a variety of human tumor types including breast cancer, prostate carcinoma, colorectal cancer, glioma and leukemia.¹³ Its overexpression has been correlated with cancer susceptibility and poor prognosis in non-small-cell lung cancer,¹⁴ oral cancer¹⁵ and anaplastic thyroid cancer,¹⁶ among others. This suggests that Aurora B kinase might constitute a drug target for anticancer chemotherapy.

Several Aurora kinase inhibitors including ZM447439,^{17,18} AZD1152,¹⁹ PHA-680632,²⁰ and MK0457/VX-680,²¹ have been developed. AZD1152 is a novel, highly-potent and specific aurora kinase inhibitor with selectivity for Aurora B kinase (Ki 0.4 nM). AZD1152 displays a high level of specificity, as determined on a panel of 50 additional serine-threonine and tyrosine kinases. *In vitro* treatment of human tumor lines with AZD1152-HQPA (the active drug in human plasma) suppresses the phosphorylation of

*Correspondence to: Guido Kroemer and Eric Deutsch; Email: kroemer@igr.fr and deutsch@igr.fr

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the Aurora B substrate histone H3. AZD1152-HQPA displays a novel mechanism of action as it affects chromosome alignment and segregation. AZD1152-treated cells become polyploid due to failed cell division before they die.²²

In radiochemotherapy, drugs are combined with radiation with the goal to achieve therapeutic effects that are superior to those obtained with the single drug or radiation alone.²³ We have recently shown that AZD1152 enhances the effect of irradiation on p53-deficient cancer cells.¹⁹ This effect was also observed when Aurora B was depleted with a small interfering RNA (siRNA) or inhibited by transfection with an inducible kinase-dead Aurora B mutant. AZD1152 increased radiation-induced cell killing in several cancer cell lines *in vivo*, especially in p53-deficient xenografts that were implanted into immunodeficient mice.¹⁹ In clinical practice, radiation therapy and chemotherapy are combined in three distinct schedules: neoadjuvant (drug before radiation), adjuvant (drug after radiation), and concomitant (drug used at the same time as radiation). Here, we have addressed the question: in which schedule of chemoradiotherapy would the radiosensitizing effect of Aurora B inhibition be optimal? The results of this preclinical study underscore the importance of mitotic catastrophe for the radiosensitizing activity of AZD1152.

Results

Time sequence-dependent effects of ionizing radiation and AZD1152-HQPA on cancer cells *in vitro*. We have previously shown that AZD1152 can enhance the effect of irradiation *in vitro* and *in vivo*, especially in cancer cells that lack p53 or that carry mutant, inactive p53 protein. Here, we evaluated the influence of the temporal order (neoadjuvant vs. adjuvant vs. concomitant) of AZD1152 and irradiation on cancer cells *in vitro*. For this, p53^{-/-} HCT116 colon cancer cells were exposed to 50 nM AZD1152-HQPA and irradiated with 10 Gy, either immediately after AZD1152-HQPA addition, or later (at 4, 8 or 24 h) and collected 24 h after IR. In the absence of IR, AZD1152-HQPA induced a large population of p53^{-/-} HCT116 cells to accumulate a DNA content >4N indicative of polyploidy. The >4N population was much reduced when ionizing radiation (IR) was administered concomitantly with AZD1152-HQPA, but was augmented when the time interval between AZD1152-HQPA and IR increased (Fig. 1A). When p53^{-/-} HCT116 cells were first irradiated and then exposed to AZD1152-HQPA at 0, 4, 8 (not shown) or 24 h (Fig. 1B), a similar effect was observed as in the neoadjuvant schedule (AZD1152 followed by IR). As the interval between IR and AZD1152-HQPA increased, the percentage of >4N cells increased (Fig. 1B). Immunofluorescence staining with the MPM2 antibody (a mitosis marker) showed that AZD1152-HQPA increased the population of >4N cells but did not increase the percentage of mitotic cells, and concomitant combination of IR and AZD1152-HQPA did not change the percentage of mitotic cells, although the population of >4N cells was greatly reduced (Fig. 1C).

Clonogenic survival assays confirmed that the efficacy of AZD1152-HQPA and irradiation was dependent on the temporal order of both treatments. The addition of AZD1152-HQPA

failed to increase the radiosensitivity in HCT116 cells when the drug was applied concomitantly with irradiation (33% surviving cells) (Fig. 1D). In contrast, sequential treatment of 10 nM AZD1152-HQPA 24 h before or after IR (2 Gy) led to a clear reduction in clonogenic survival after IR (15 or 11% surviving cells) compared with IR alone (25% surviving cells). This indicates that the administration of AZD1152-HQPA 24 h before or after IR has a radiosensitizing effect (Fig. 1D).

Schedule-dependency of AZD1152 on radiation response *in vivo*. We have previously shown that 4-day administration of AZD1152 (35 mg/kg/day) increased radiation-induced tumor cell killing compared with IR alone in mice bearing subcutaneous p53^{-/-} HCT116 colon cancer xenografts.¹⁹ Here, we compared three different schedules of AZD1152 (4-day dosing of 35 mg/kg/day) and radiation: a neoadjuvant schedule (AZD1152 on days 1–4, followed by one dose of 8 Gy radiation on day 5); an adjuvant schedule (one dose of 8 Gy radiation on day 1 followed by AZD1152 on days 2–5); and a concomitant schedule (AZD1152 on days 1–4 combined with one dose of 8 Gy radiation on day 2). We then determined the number of days for the tumors to reach a volume of 800 mm³ following IR. In the IR-only group, a tumor volume of 800 mm³ was reached after 32 days. Confirming the *in vitro* results (Fig. 1), we found that the neoadjuvant schedule, (AZD1152 pre-irradiation, Fig. 2A) and the adjuvant schedule (AZD1152 post-irradiation, Fig. 2B, Table 1) resulted in a significant delay in tumor growth and a tumor volume of 800 mm³ was reached as late as on day 37. In contrast, tumor growth was similar in mice treated with the concomitant schedule or IR alone (Fig. 2C).

Combination of fractionated radiation and AZD1152 *in vivo*. Given the fact that radiation therapy is routinely fractionated in the clinics, mostly by repeated daily radiation doses, we evaluated the effects of AZD1152 (AZD1152 35 mg/kg/day *i.p.* for 4 days) on the fractionated radiation response (3 Gy for 4 days at the same time as the drug) *in vivo*. AZD1152 improved the delay in tumor growth when it was combined with fractionated radiation (time to reach a tumor volume of 800 mm³: 33 days vs. 28 days in the AZD1152 plus IR vs. IR groups, respectively) (Fig. 2D, Table 1). In previous studies of combination therapy, AZD1152 has been administered for 4 days.¹⁹ To determine whether the tumor growth delay observed *in vivo* was dependent on the schedule of AZD1152 administration, we administered one single dose of AZD1152 (160 mg/kg) followed by IR (8 Gy, 1 day later) to mice bearing p53^{-/-} HCT116 tumors. Although this single dose of AZD1152 did retard tumor growth, it failed to mediate a significant radiosensitizing effect (Fig. 2E).

AZD1152 induces mitotic catastrophe *in vivo* in HCT116 tumor xenografts. As AZD1152 induced polyploidy in a number of distinct cell lines *in vitro*, we wondered whether this Aurora B kinase inhibitor might cause mitotic catastrophe. Mitotic catastrophe or ‘mitotic cell death’ is considered as a type of cell death that is caused by altered mitoses and/or irreparable chromosomal damage.^{24–26} Mitotic catastrophe occurs during or shortly after a deregulated or failed mitosis and can be accompanied by morphological alterations such as micronucleation (the appearance of chromosomes or chromosomal material outside of

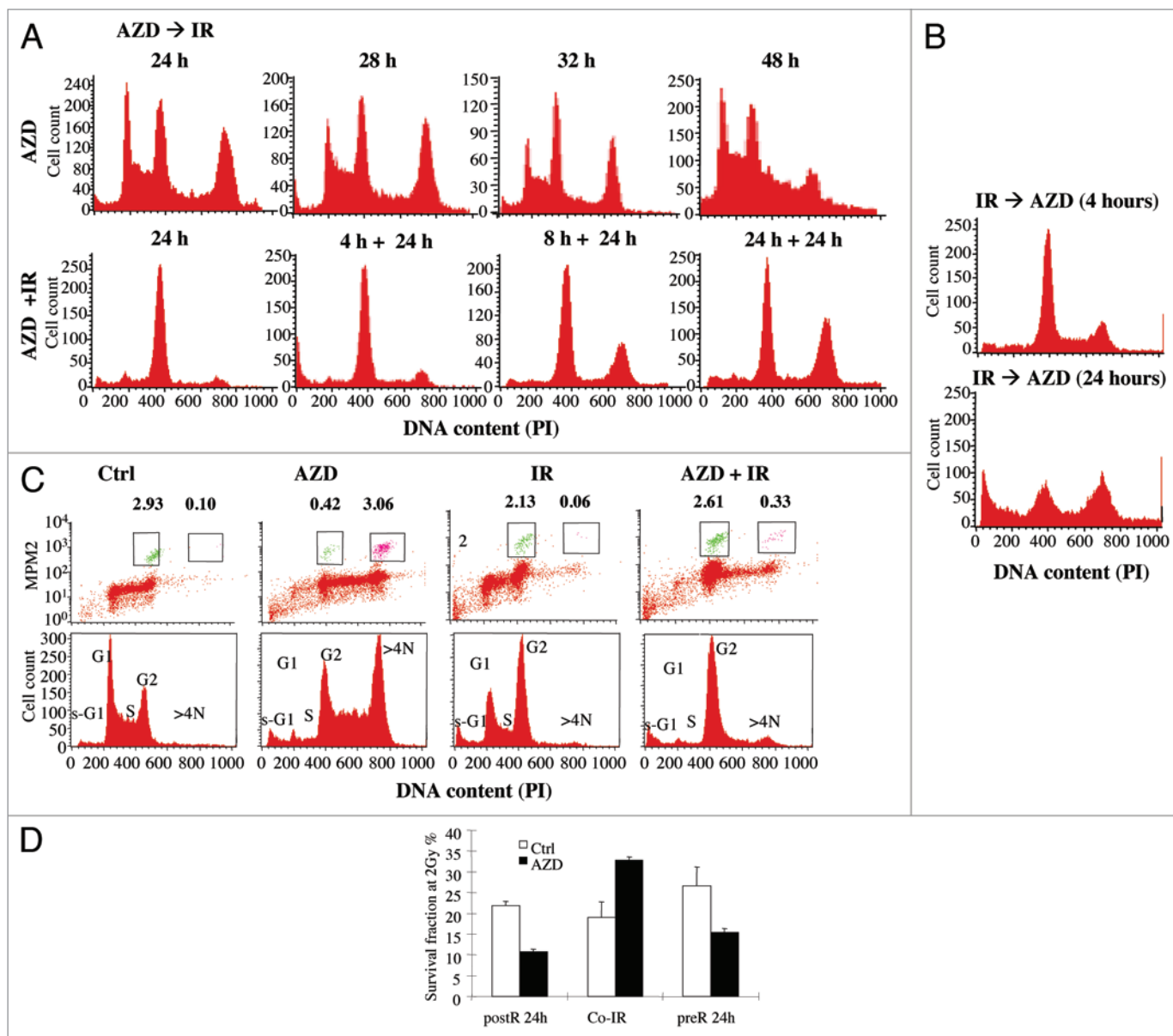


Figure 1. Time sequence dependence of the antineoplastic effects of AZD1152-HQPA and IR in vitro. (A) Effect of different schedules of AZD1152-HQPA with ionizing radiation (IR) on the cell cycle of p53^{-/-} HCT116 cells. Cells were exposed to 50 nM AZD1152-HQPA (0, 4, 8 or 24 h) followed by IR (10 Gy, lower images) or AZD1152-HQPA alone (upper images), and collected 24 h later for cell cycle analysis. The population of >4N cells was dramatically reduced when cells were treated concomitantly with AZD1152-HQPA and IR. The population of >4N cells increased, as the time interval between AZD1152-HQPA and IR increased (AZD1152-HQPA 4, 8 or 24 h + 10 Gy 24 h). Data from one representative experiment out of three independent experiments (performed in triplicate) are shown. (B) Cells treated with IR 10 Gy for 4 (10 Gy 4 h + AZD1152-HQPA 24 h) or 24 h (10 Gy 24 h + AZD1152-HQPA 24 h) followed by additional 24 h exposure of AZD1152-HQPA (50 nM). Cell cycle analysis showed that, as the interval between IR and AZD1152-HQPA increased, the percentage of >4N cells increased. (C) Mitotic cell analysis in p53^{-/-} HCT116: FACS analysis using a MPM2 antibody as a mitotic marker. Cells were exposed to AZD1152-HQPA (50 nM) for 24 h, or to 10 Gy alone (IR), or AZD1152-HQPA followed immediately by 10 Gy (AZD + IR). Cells were fixed 24 h after IR. (D) Time sequence-dependent clonogenic cell survival of AZD1152-HQPA and IR combination in HCT116 cells: cells were treated with AZD1152-HQPA (10 nM) at the same time as IR 2 Gy (Co-IR) or 24 h after IR (postR 24h) or 24 h before IR (preR 24h). Data (means ± SEM) from one representative experiment out of three independent experiments (performed in triplicate) are shown.

the two daughter nuclei) and multinucleation (the appearance of two or more nuclei similar or heterogeneous in size, resulting from deficient separation during cytokinesis).²⁷⁻²⁹ First, we studied the nuclear changes induced by AZD1152 in vivo. Mice harboring p53^{-/-} HCT116 colon cancer xenografts were treated with AZD1152 (35 mg/kg/day for 4 days,) or vehicle, and tumor

samples were recovered 24 h, 48 h or 3 weeks (Fig. 3A and B) after treatment. At all time points we found that AZD1152 (but not the vehicle) induced the accumulation of multinucleate cells and micronuclei. This effect was even more pronounced when AZD1152 was combined with IR (Fig. 3C and D), suggesting that the combination induces cell death by mitotic catastrophe.

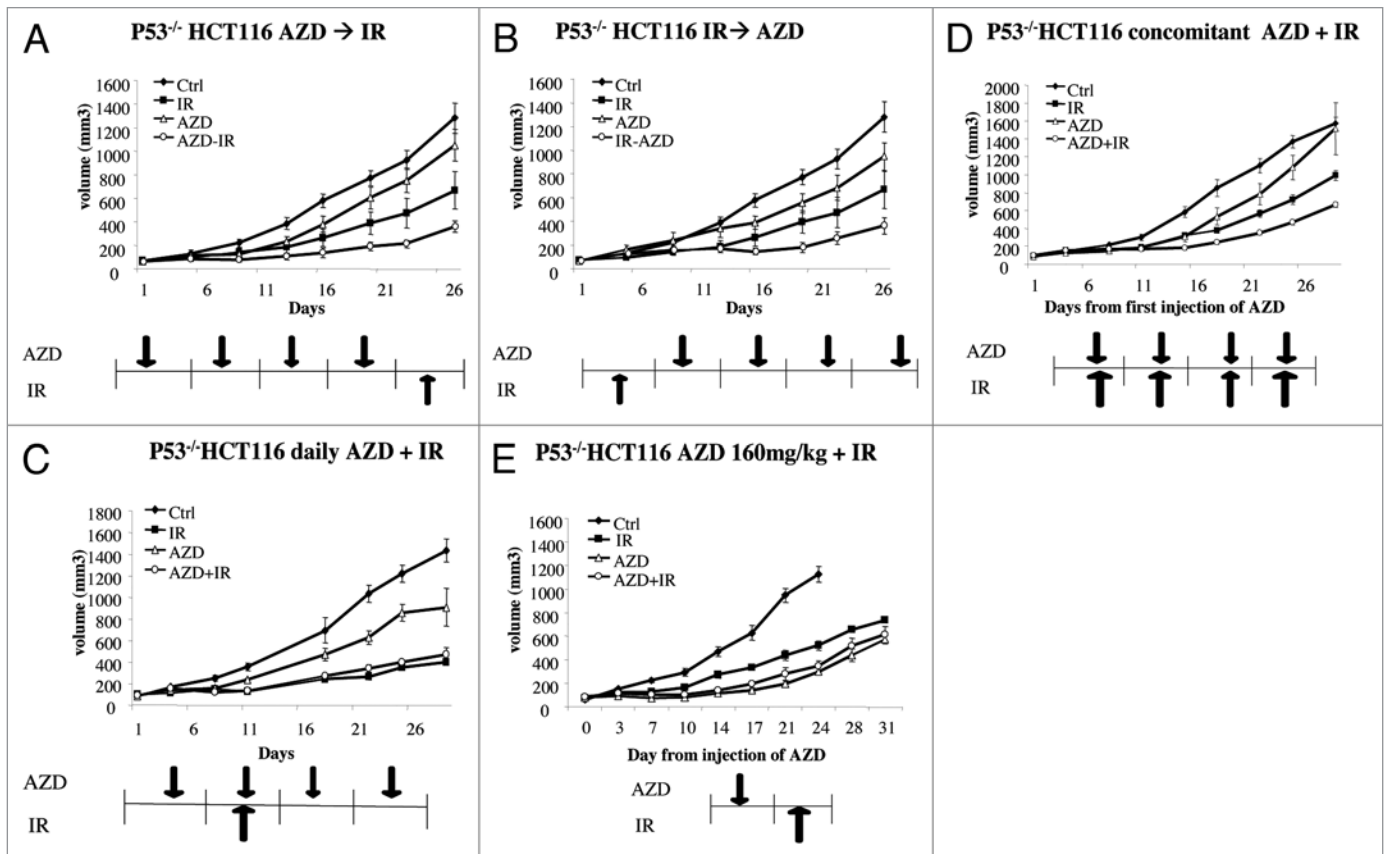


Figure 2. Schedule-dependency of AZD1152 on radiation response in vivo. Mice inoculated with subcutaneous p53^{-/-} HCT116 xenografts were treated with different schedules of AZD1152 (35 mg/kg/day i.p. for 4 days) and IR (8 Gy, one fraction). Upper panels represent tumor volume. (A) Neo-adjuvant schedule (AZD1152 on days 1–4, followed by one dose of 8 Gy radiation on day 5); (B) adjuvant schedule (one dose of 8 Gy radiation on day 1 followed by AZD1152 on days 2–5); and (C) concomitant schedule (AZD1152 on days 1–4 combined with one dose of 8 Gy radiation on day 2). n = 6 per group, mean tumor volumes ± SEM are shown. Combination of fractionated radiation and AZD1152 in vivo shown in (D): Tumor growth in mice treated with AZD1152 (35 mg/kg/day i.p. for 4 days) and fractionated radiation (3 Gy for 4 days concomitantly delivered with AZD1152, days 1–4). n = 6 per group, mean tumor volumes ± SEM are shown. (E) Tumor growth in mice treated with AZD1152 160 mg/kg on day 1 followed by 8 Gy IR on day 2. Mean tumor volumes ± SEM (n = 6) are shown.

Table 1. Tumor growth delays in the AZD1152 + IR versus IR alone in the various schedule tested

	Time to reach 800 mm ³ AZD1152 + IR vs. IR	Difference
Neo-adjuvant AZD1152 single dose IR	37 days vs. 32 days	5 days
Adjuvant AZD1152 single dose IR	37 days vs. 32 days	5 days
Concomitant AZD1152 single dose IR	32 days vs. 32 days	0 day
Concomitant AZD1152 fractionated IR	33 days vs. 28 days	5 days

AZD1152 induces mitotic catastrophe in vitro. We used time-lapse videomicroscopy to detect multi- and micronucleation in real time. RKO colon cancer cells that had been stably transfected with a histone H2B-GFP fusion construct (that allows for the visualization of chromosomes as a green fluorescence), were treated with AZD1152-HQPA in the absence or presence

of pifithrin- α , a chemical inhibitor of p53,¹⁹ followed by videomicroscopy in the presence of propidium iodide (to detect events of plasma membrane permeabilization; Fig. 4A). These observations revealed AZD1152-induced micro- and multinucleation, which was often followed by cell death (Fig. 4A). A similar effect could be seen when AZD1152-HQPA was added to HCT116 cells (Fig. 4B).

As AZD1152-HQPA-treated cells develop a significant fraction of polyploid cells (DNA content >4N), we FACS-sorted the population of >4N cells after exposure to AZD1152-HQPA in order to assess their clonogenic survival in comparison with their non-polyploid counterparts. Cells were separated by FACS analysis according to their DNA content into >4N and <4N cells and then plated into fresh medium to assess their capacity to form clones after 12–14 days of culture. Polyploid cells displayed a significantly lower clonogenic survival than the <4N cells in HT29 and RKO cell lines (8% vs. 32% for HT29 and 17% vs. 80% for RKO, respectively; Fig. 5A and B). These results support the hypothesis that polyploidy induced by AZD1152-HQPA can constitute a lethal event.

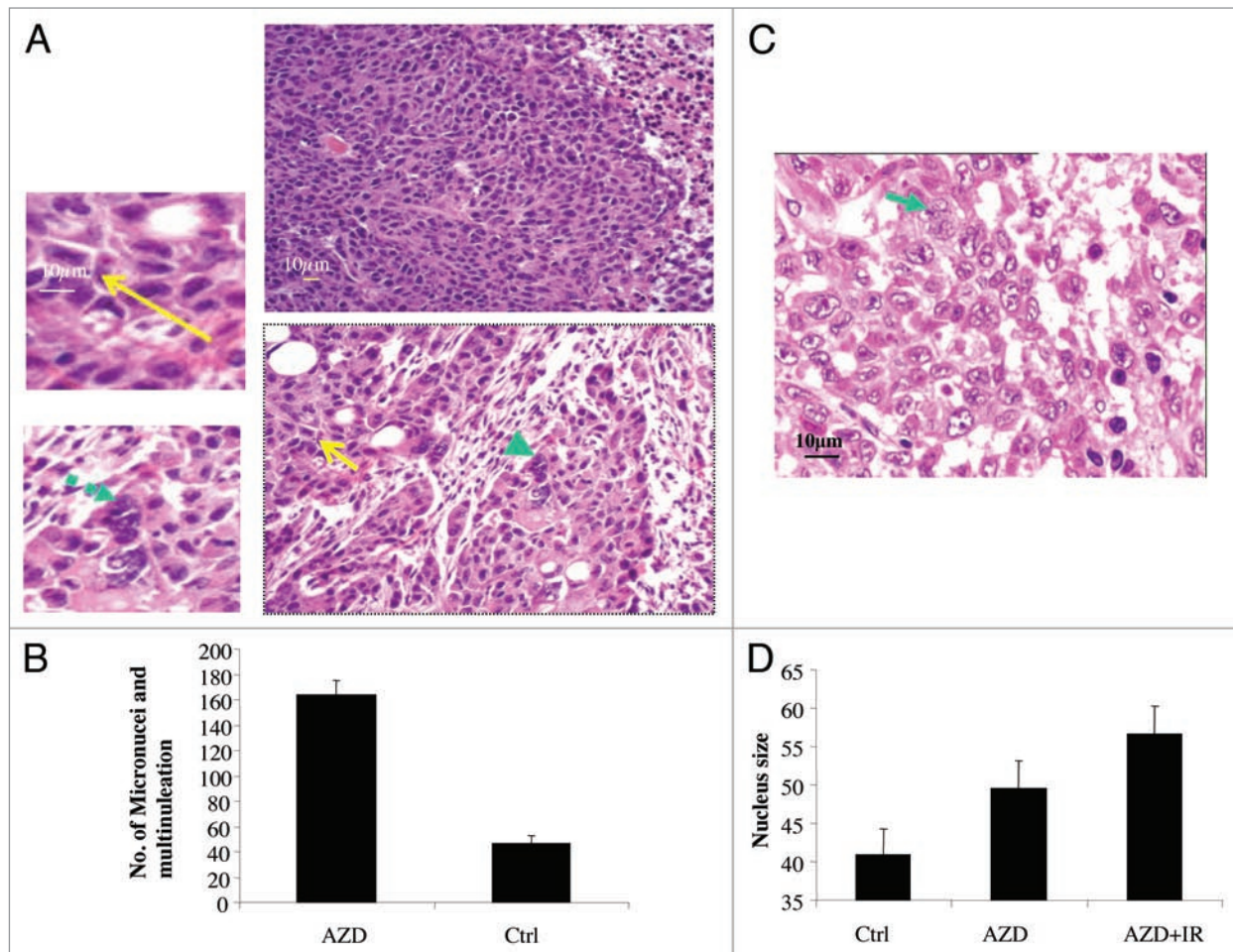


Figure 3. Cells with multinucleation and micronuclei (indicating mitotic catastrophe) were induced *in vivo* by AZD1152 in a subcutaneous p53^{-/-} HCT116 xenograft model. (A) HE-stained histological sections. In the upper right panel, images of control cells treated with vehicle are shown. In the lower right panel, images of p53^{-/-} HCT116 tumor xenografts treated with AZD1152 (35 mg/kg/day for 4 days) are shown. Tumors were collected 3 weeks after treatment. In the AZD1152-treated tumor, anisokaryosis was observed, with numerous multinucleate cells. The yellow arrow (upper left) indicates micronuclei; the green arrow (lower left), a multinucleate cell. (B) comparison of number of cells with micronuclei or multinucleation per 10 microscopic high-power fields (x400) in tumor xenografts treated for 24 h with AZD1152 or no treatment as control. (C) Histological section of p53^{-/-} HCT116 xenograft after treatment with 80 mg/kg AZD1152 and 8 Gy IR. The arrow indicates a multinucleate cell. (D) comparison of the mean size (surface) of cell nuclei in p53^{-/-} HCT116 cells xenograft tumors. Nuclei from 50 cells were measured in each tumor. Error bars represent SD.

Apoptosis following exposure to AZD1152-HQPA. We observed a modest increase in cells that bind an Annexin V-FITC conjugate, 24–72 h after the addition of AZD1152-HQPA to HCT116 cells or RKO cells (data not shown), suggesting a minor induction of apoptosis. Apoptosis is characterized by a characteristic accumulation of biochemical changes, in particular mitochondrial outer membrane permeabilization (that is regulated by proteins from the Bcl-2 family including the proapoptotic protein Bax) coupled to the activation of a specific class of cysteine proteases cleaving after Asp residues, the caspases.³⁰ Therefore, we compared the clonogenic survival of apoptosis-competent HCT116 WT and apoptosis-resistant HCT116 Bax^{-/-} cells exposed to AZD1152-HQPA. The absence of Bax did not affect clonogenic death induced by the Aurora kinase B inhibitor (Fig. 6A). We also determined the potential cytoprotective effects of the pan-caspase inhibitor Z-Val-Ala-DL-Asp (OMe)-fluoromethylketone (Z-VAD-fmk). HCT116 cells were simultaneously treated with

Z-VAD-fmk and AZD1152-HQPA or AZD1152-HQPA alone for 24 h, followed by determination of the clonogenic survival in the absence of both agents. Again, Z-VAD-fmk failed to improve the clonogenic potential of AZD1152-HQPA-treated cells (Fig. 6B, left). A similar result was obtained when HCT116 cells were stably transfected with the Baculovirus-derived p35 inhibitor of apoptosis protein, a pan-caspase inhibitor (Fig. 6B, right). While p35 strongly inhibited apoptosis induced by cytotoxic agents such as staurosporin, cisplatin and camptothecin,³¹ it failed to rescue cells from AZD1152-HQPA-induced replicative cell death. In conclusion, it appears that neither pro-apoptotic Bcl-2-like proteins nor caspases are required for AZD1152-HQPA-induced cell killing.

Contribution of the G₂-M checkpoint in AZD1152-HQPA-mediated cell death. We have shown previously that the changes in radiosensitivity induced by AZD1152 are cell cycle dependent. We took advantage of two HCT116 derivatives with weakened

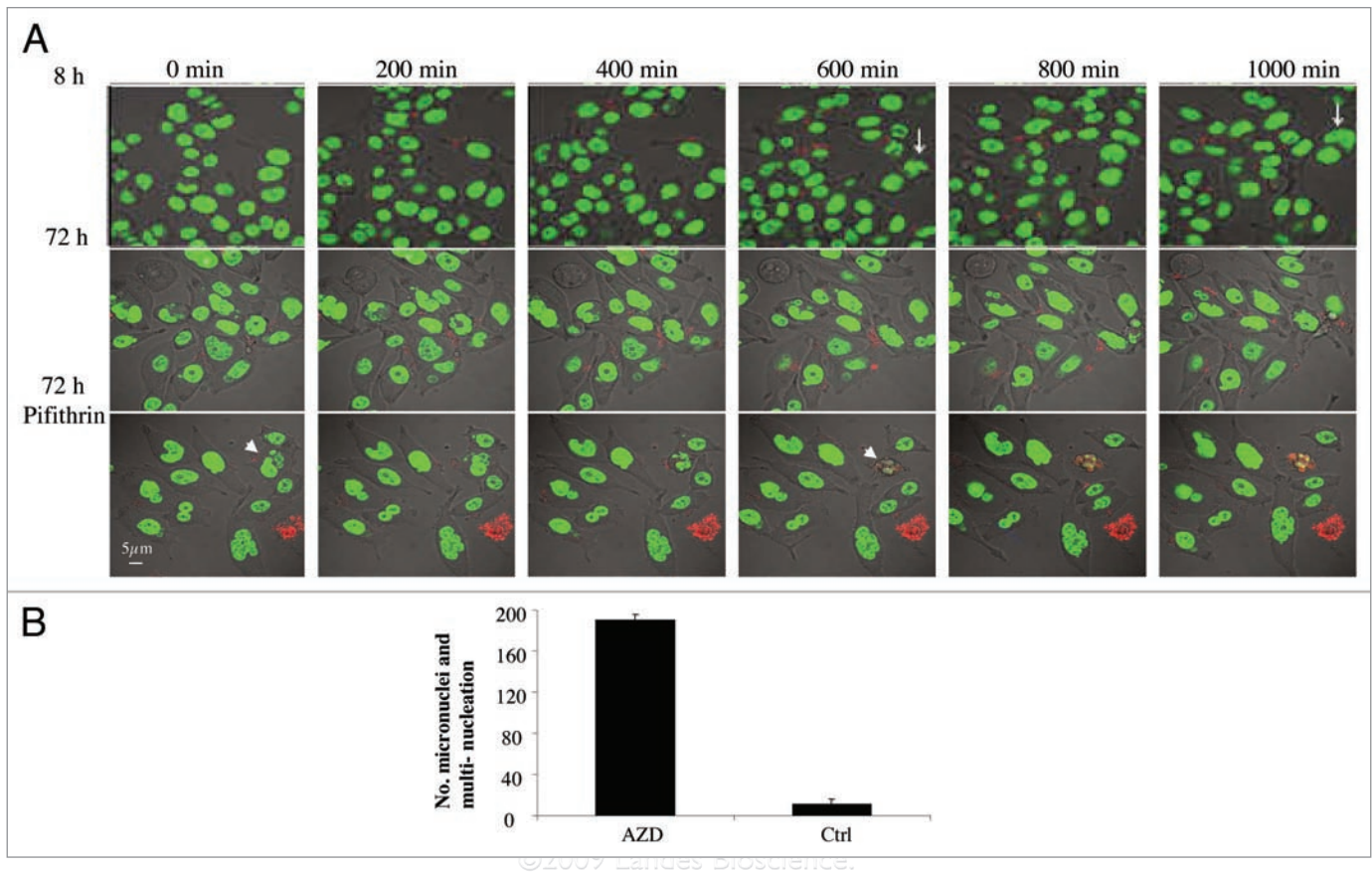


Figure 4. AZD1152 induces mitotic death. (A) Time lapse confocal videomicroscopy after treatment with AZD1152-HQPA. RKO colon cancer cells were stably transfected with a histone H2B-GFP fusion construct that allows visualization of chromosomes as a green fluorescence. The vital dye PI (excluded from live cells, red fluorescence) was added prior to confocal microscopy. Upper panel: cells were treated with AZD1152-HQPA (50 nM for 8 h), micronucleation occurred after mitosis (indicated by the arrow). Middle panel: cells were treated with AZD1152-HQPA for 72 h. Most cells have micronuclei or are multinucleated. Bottom panel: cells were treated with pifithrin- α (a chemical inhibitor of p53) for 8 h before additional 72 h exposure of AZD1152-HQPA. The arrow indicates one multinucleated cell incorporating PI. The video was run for a total of 20 h following each treatment. (B) The number of HCT116 cells with multinucleation and micronuclei increased after exposure to AZD1152-HQPA (50 nM). Nuclei were stained with Hoechst 33342. The percentage of cells with multinucleation and micronuclei were counted among a total of 200 cells. Data represent the mean \pm SD of three independent experiments.

cell cycle checkpoints (14-3-3 σ ^{-/-} and CHK2^{-/-}) as well as a specific pharmacologic inhibitor of checkpoint kinase 1 (CHK1) to determine whether the G₂-M checkpoint affects the cytosidal activity of AZD1152-HQPA.

14-3-3 σ ^{-/-} (but not CHK2^{-/-}) cells were hypersensitive to AZD1152-HQPA, compared with WT HCT116 cells (Fig. 6C). The CHK1 inhibitor, Chir124, also increased the sensitivity of the WT cells (Fig. 6D) to AZD1152-HQPA. This suggests that in the context of CHK1 or 14-3-3 σ deficiency, cells could be more likely to override the G₂-M checkpoint and hence become more susceptible to the induction of mitotic death by AZD1152-HQPA. We tested this hypothesis by FACS-purifying RKO-GFP cells into G₁-S (2N) and G₂-M (4N) populations which were then treated with 50 nM AZD1152-HQPA for 24 h and then subjected to clonogenic assays. Importantly, G₂-M cells were much more sensitive to AZD1152-HQPA than G₁-S cells (data not shown), underscoring the connection between mitosis and cell death.

AZD1152-HQPA does not affect γ H2AX DNA damage foci. We monitored the appearance of IR-induced γ H2AX foci by in

situ immunofluorescence or cytofluorometry in WT HCT116 or p53^{-/-} HCT116 cells. Cells were treated with AZD1152-HQPA (50 nM) for 24 h and then irradiated with 2 Gy (for in situ immunofluorescence) or 10 Gy (for FACS analyses). Then cells were collected at different time points (0 min, 15–20 min, 2, 6 and 24 h), fixed and labeled for the detection of γ H2AX. AZD1152-HQPA failed to affect the frequency of irradiation-elicited γ H2AX foci in WT and p53^{-/-} HCT116 cells, both in their appearance (which reflects acute DNA damage, e.g., 20 min post-irradiation) and their disappearance kinetic (which reflects DNA repair) (data not shown). To further elucidate the physiological contribution and potential mechanism of Aurora B, we employed a tetracycline inducible kinase-dead (dominant-negative, DN) Aurora B mutant that has been stably transfected into HEK293 cells.³² The tetracycline-induced overexpression of WT Aurora B or DN Aurora B did not affect the appearance or disappearance of γ H2AX foci induced by IR (data not shown). Altogether, these results suggest that AZD1152, as well as its target Aurora-B, do not affect the kinetics of DNA DSB repair after DNA damage.

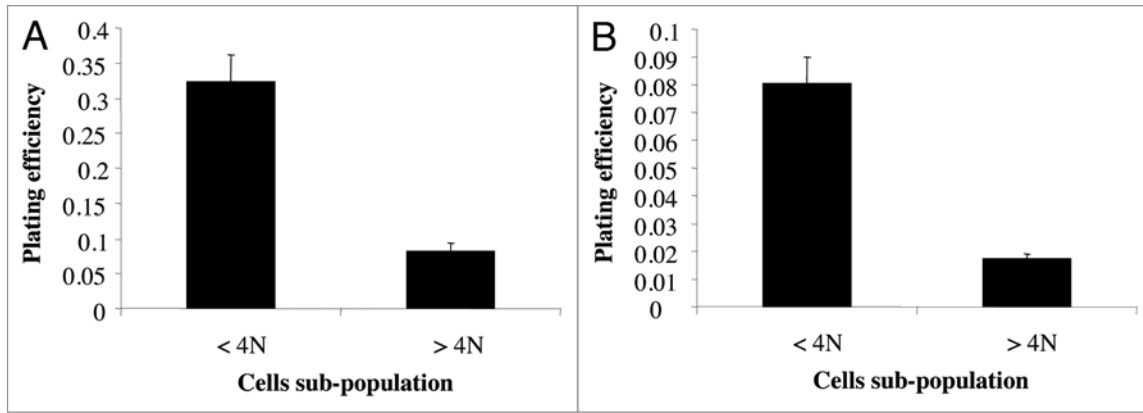


Figure 5. (A) After 24 h of AZD1152-HQPA exposure (50 nM), HT29 cells were sorted by FACS into >4N and <4N cells according to the DNA content and then transferred into fresh medium to determine colony formation within 12–14 days of culture. Clonogenic survival curves in >4N cells and <4N cells are shown. (B) RKO colon cancer cells were stably transfected with a histone H2B-GFP fusion construct, treated with AZD1152-HQPA (50 nM for 24 h) and then separated into >4N and <4N cells according to their DNA content. Subsequently, cells were cultured in fresh medium to determine colony formation within 12–14 days.

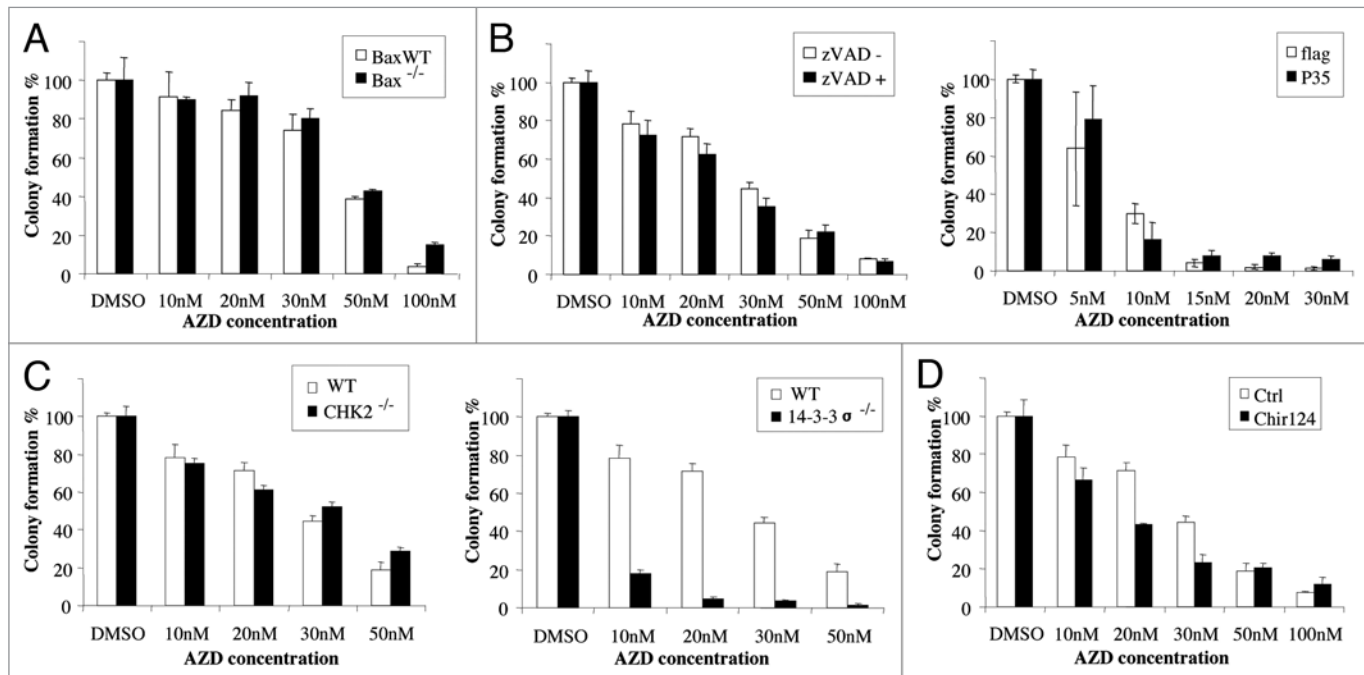


Figure 6. Cell sensitivity to AZD1152 is not affected by apoptosis inhibition. (A) Impact of Bax. Colony formation assays were compared in wild type (WT) and Bax^{-/-} HCT116 cells. (B) Impact of caspases. Colony formation was determined for WT HCT116 exposed to the pan-caspase inhibitor z-VAD-fmk (left). Alternatively, flag (control) transfected HCT116 cells were compared to cells stably transfected with the Baculovirus-derived inhibitor of apoptosis protein, p35, a pan-caspase inhibitor (right) (C and D). Contribution of the G₂-M checkpoint in AZD1152-mediated cell death. Clonogenic survival assays of two HCT116 cell lines in response to AZD1152-HQPA at different concentrations. CHK2^{-/-} HCT116 cells were compared with WT HCT116 cells (C, left) or 14-3-3^σ ^{-/-} HCT116 were compared to WT HCT116 cells (C, right). WT HCT116 cells were treated with or without 250 nM Chir124 (a selective CHK1 inhibitor) for 1 h followed by a further 24 h exposure to AZD1152-HQPA (D). Data from one representative experiment out of three independent experiments (performed in triplicate) are shown as means ± SEM.

Discussion

More than ten different Aurora kinase inhibitors have been explored in preclinical studies and some of them, including AZD1152,¹⁹ MLN8054,³³ and PHA-739358,³⁴ are now being

investigated in clinical trials. Recently, we have demonstrated the capacity of AZD1152 to enhance the effects of IR, both in vitro and in vivo, in human cancer xenograft models.¹⁹ Nonetheless, the optimal sequence of AZD1152 and IR combination therapy remains undefined. In this study, we investigated different

schedules of AZD1152 and radiation. Our *in vitro* and *in vivo* data indicate that appropriate scheduling of Aurora B kinase inhibition and IR is important for the efficacy of the combination. The effect of AZD1152 on IR response depends on the temporal order between the application of the drug and IR, as is the case for other drugs that target mitosis, such as paclitaxel and docetaxel.³⁵ We observed enhanced cell killing when cells were irradiated 24 h after exposure to AZD1152, whereas the concomitant application of IR and AZD1152 failed to yield an additive or synergistic effect. As fractionated radiotherapy is mostly used in the clinic, we also investigated the effects of AZD1152 combined with fractionated IR. Of interest, fractionated IR + AZD1152 led to an improved radiation response *in vivo* that was in the same range as that obtained with a single IR dose. This is important for the transfer of our preclinical data into a clinical trial.

The precise mechanism of tumor cell killing induced by AZD1152 remains to be clarified. We previously demonstrated that IR can induce multi- and micronucleation coupled to mitotic catastrophe, the predominant cell death type after IR.³¹ Some authors have suggested that the inhibition of Aurora kinases would induce apoptosis.³⁶ Here, we found that although AZD1152 induced a low frequency of acute apoptotic events, the long-term (clonogenic) cell death induced by AZD1152 was not dependent on apoptotic regulators such as Bax and caspases. This is in contrast to another study³⁷ that showed that in response to KSP (kinesin-5, a mitotic spindle motor protein) inhibition, activation of the spindle checkpoint was followed by mitotic slippage, which in turn initiated apoptosis by activating Bax. One cannot rule out the possibility that inhibition of KSP and Aurora B kinase might unleash different cell death mechanisms. Moreover, assays that determine long-term cell death (by clonogenic assays) and immediate cell death (by detecting signs of apoptosis) may yield divergent results, because inhibition of acute cell death does not necessarily avoid the loss of clonogenic survival. AZD1152 inhibits Aurora B kinase, thereby antagonizing the spindle checkpoint and abolishing cytokinesis. Our data raise the possibility that abolition of spindle assembly checkpoint (by AZD1152 in the present study) combined with DNA damage (by IR in the present study) may constitute a general strategy to promote mitotic catastrophe. We also demonstrated that multi- and micronucleation induced by AZD1152 plus IR is predictive of later cell death, as indicated by videomicroscopic observations and the assessment of the clonogenic survival of polyploidy cells. These results suggest that the histochemical evidence of multi- and micronucleation may be interpreted as a sign of therapeutic response and hence may be tentatively considered as a biomarker for the clinical monitoring of AZD1152 effects.

In conclusion, our results demonstrate that different schedules of administration of AZD1152 and radiation can enhance radiation-induced cell death in p53-deficient cancer cells *in vivo*. Apoptosis and DNA repair play a minor role in the response of tumor cells exposed to AZD1152. In contrast, mitotic death is the major cell death mechanism following AZD1152 exposure, both *in vitro* and *in vivo*. These preclinical data support further clinical exploration of AZD1152 in combination with fractionated radiotherapy.

Materials and Methods

Cell lines. HCT116 human colorectal cancer cell lines p53 wild type [WT], p53^{-/-},³⁸ 14-3-3 σ ^{-/-},³⁹ CHK2^{-/-},⁴⁰ were a kind gift from B. Vogelstein (Johns Hopkins University, Baltimore, MD, USA). The HT29 colorectal cancer cell line (with p53 and Raf mutation) was obtained from the American Type Culture Collection (Manassas, VA, USA). HCT116 and HT29 cells were maintained in McCoy's 5a medium (Gibco) supplemented with 10% fetal bovine serum (ATGC), 1% PS (Gibco), 1% L-glutamine (Eurobio), 1 mM sodium pyruvate (Gibco) and 10 mM HEPES (Sigma). RKO colon cancer cells, transfected with a histone H2B-green fluorescent protein (GFP) fusion construct, were cultured in the same medium supplemented with 20 mg/ml supplementary blasticidin (Invitrogen).

To generate p35 clones, the HCT116 parental cells were transfected with a vector encoding p35, selected with geneticin (0.5 mg/ml, Invitrogen, Cergy-Pontoise, France) and subcloned by limiting dilution. As control, HCT116 parental cells clones were transfected with a pCMV vector (Sigma) that confers resistance to geneticin.

Stable, isogenic cell lines expressing Aurora B transgenes (stable cell lines expressing the WT Aurora B kinase were generated, as well as transgenes harboring point mutants designed to inhibit catalytic activity. The aspartic acid in the highly conserved DFG motif was separately mutated, subdomain VII, to asparagine [D-N]) under tetracycline control were generated using the FRT-Flp-based system as previously described.³² Cell culture conditions were as described previously.³²

Cell cycle analysis. Sham control and 6 Gy-irradiated cells with or without drug exposure were harvested at the indicated time points after irradiation, fixed in 70% ethanol and stored at -20°C. Prior to DNA analysis, DNA content was labeled with 0.1 mg/ml propidium iodide (PI) and 1 mg/ml RNase. Cell cycle analysis was performed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA).

Mitotic index analysis. The cells were washed once with PBS and fixed in 70% ethanol until the day of FACS analysis. On the day of FACS analysis, the cells were incubated for one hour at room temperature with 5 μ g/ml MPM2 (1:200). The cells were incubated with secondary antibody (1:300: Alexa 488-conjugated IgG). DNA content was labeled with PI. Analysis was performed by flow cytometry.

FACS γ H2AX analysis. Cells were fixed in 70% ethanol until the day prior to FACS analysis. On the day of analysis, cells were permeabilized in 0.5% Triton for 5 min and incubated with γ H2AX antibody for 2 h. Subsequently, cells were washed and then incubated with 0.5 μ l of Alexa 488-conjugated IgG (1:200) for 1 h, stained with PI for FACS analysis.

Purification of selected cell cycle subpopulations. 24 h after incubation with AZD1152-HQPA, HT29 cells were stained with 10 mg/ml of Hoechst 33342 (Molecular Probes, Eugene, USA) for 30 min at 37°C. Samples were resuspended in medium containing 0.5 mg/ml PI. Selection of >4N and <4N cells was performed using a FACS Vantage SE (BD Biosciences). RKO-GFP cells treated with AZD1152-HQPA for 24 h were resuspended in

medium containing 0.5 mg/ml PI. Selection of G₂-M and G₁-S phase cells as well as >4N and <4N cells was performed using a FACS.

Clonogenic survival assays. Cells were seeded in triplicate into 6-well plates in a range of 100 to 80,000 cells/well, so as to yield 20–200 colonies per well. A single dose of irradiation with or without drug was applied once cells were attached. Cells were cultured in a 37°C, 5% CO₂ incubator for 10–14 days. Individual colonies (>50 cells/colony) were fixed and stained with a solution containing crystal violet and methanol for 20 min. The plating efficiency (PE) represents the percentage of cells seeded that form colonies under a specific culture condition of a given cell line. The survival fraction, expressed as a function of irradiation, was calculated as follows: Survival fraction = colonies counted/(cells seeded x PE/100). 200 KV X-ray at a dose rate of 0.66 Gy/min was used for the in vivo experiments and 137 Cesium source at a dose rate of 1.85 Gy/min was used for in vitro experiments.

Inhibition studies. The Aurora B kinase inhibitor, AZD1152 (provided by AstraZeneca Pharmaceuticals) is a phosphate pro-drug that is converted in vivo to the active moiety; AZD1152-HQPA was used in in vitro studies. AZD1152-HQPA was dissolved in DMSO (final concentration of DMSO did not exceed 0.1%) and cells were incubated with AZD1152-HQPA or DMSO vehicle control (if not specified cells were incubated for 24 h). Chir124 was used as a selective CHK1 inhibitor to determine whether the G₂-M checkpoint affects the cytotoxic activity of AZD1152. Chir124 (250 nM) was co administered with AZD1152.²⁹

In vivo xenograft in nude mice and histology. 3 x 10⁶ p53^{-/-} HCT116 cells were implanted on the right flank of each mouse. Treatment commenced when the tumor was at least 5 mm in diameter. Mice were randomly allocated to four groups: (A) control; (B) IR alone; (C) AZD1152 alone, 35 mg/kg/day for 4 days or one single dose 160 mg/kg; (D) AZD1152 combined with IR. Drug or vehicle control was administered intraperitoneally. Tumor size was measured twice a week using an electronic caliper. Tumor volume was estimated from two-dimensional tumor measurements by the formula: Tumor volume = length (mm) x width² (mm²)/2 (six or eight mice per group in each group). Tumor assessments were performed over 30–60 days or until a mean tumor volume of at least 800 mm³ was reached. Subsequently, mice were sacrificed and tumors

collected. Tumors were fixed in AFA and sectioned. Histological sections were stained with hematoxylin-eosin (HE). Number of multinucleated cells was evaluated at 10 randomly chosen high-power fields (x400). Measurement of nuclei surfaces was made using Cell-D Imaging Software from Olympus. For each tumor, 150 nuclei were measured on 4–6 randomly chosen high-power fields (x800).

Immunocytochemical detection of micronuclei and videomicroscopy. Cells fixed with 4% paraformaldehyde (PAF; Sigma) were stained for the detection of micronuclei and multinucleation. For fluorescence microscopy, nuclei were stained with Hoechst 33342 (1 mg/ml, Molecular Probes). Micronuclei and multinucleation were checked by fluorescence microscopy and morphologically classified according to standard criteria.³¹ More than 200 cells were scored for each data point in at least three separate experiments. RKO cells expressing GFP were cultured in the 35 mm glass-bottomed culture dishes (MatTek Corporation, Ashland, USA), maintained at a constant temperature of 37°C and an atmosphere of 5% CO₂, and were subjected to pulsed observations for 20 h using a LSM 510 laser scanning confocal microscope (Zeiss, Oberkochen, Germany). Cell viability was assessed by PI staining (100 ng/ml PI was added before confocal scanning). For γH2AX foci stain: cells were saturated with 5 mg/ml BSA/PBS for 20 min after permeation, and then incubated with primary antibody γH2AX (1:600) for 1 h 30 min. Subsequently, cells were incubated with an IgG Alexa 488 conjugate (1:500) for 1 h followed by Hoechst 33342 (1 mg/ml).

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