The Topoisomerase I Poison CPT-11 Enhances the Effect of the Aurora B Kinase Inhibitor AZD1152 both in vitro and in vivo

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Abstract

Purpose: AZD1152 is an Aurora B kinase inhibitor currently in clinical trials. As the topoisomerase I poison CPT-11 induces a G2 arrest, a mechanistic understanding of the cell cycle interactions between these agents may prove critical for combination therapy.

Methods: AZD1152 was tested in vitro and in vivo with SN-38 and CPT-11 against HCT-116 cells. Inhibition of clonogenicity, induction of apoptosis, effects on polyploidy, and tumor growth were examined.

Results: AZD1152 alone induced polyploidy of HCT-116 cells at low nanomolar concentrations. The induction of apoptosis required prolonged exposure (48 hours) and higher concentrations of drug. When SN-38 was given before or concomitantly with AZD1152, SN-38 blocked the AZD1152 effect by arresting cells in G2 and inhibiting cells from undergoing polyploidy. With the reverse combination (AZD1152 followed by SN-38), there was a significant induction of polyploidy and apoptosis, even with shorter exposure (24 hours) of AZD1152. In vivo, AZD1152 alone suppressed HCT-116 xenograft tumor growth in a dose-dependent manner with target inhibition of phosphoH3, induction of multinucleated giant cells, but without induction of apoptosis. In combination, both sequences in vivo (CPT->AZD, AZD->CPT, P = 0.008, AUC/d) proved superior to either single agent therapy. However, AZD->CPT still showed a greater increase in apoptosis and greater suppression of tumor regrowth than CPT->AZD (P = 0.02, AUC/d).

Conclusions: The results from these studies indicate a promising therapeutic strategy for combining AZD1152 with CPT-11, and suggest that the sequence of drug administration is pivotal when an Aurora B kinase inhibitor is administered with a topoisomerase I poison.

Aurora kinases are mitotic serine threonine kinases. They play an important role in chromosome alignment, segregation, and cytokinesis. The Aurora kinase family consists of three members, Aurora A, B, and C kinases (1). Aurora A and Aurora B are highly homologous in sequence and structure, but share few regulators or substrates and are different in their subcellular distribution (2). Aurora A localizes to the spindle poles where it is involved mainly in centrosome separation, mitotic entry, and spindle assembly. Aurora B, which moves to centromeres from prometaphase to metaphase and relocates to the midbody until cytokinesis is completed, is important in chromosome alignment, microtubule-kinetochore attachment, spindle check point, and cytokinesis (3–5). The activity of Aurora kinases is strictly regulated during mitosis, with Aurora A activity peaking at the G2-M transition, whereas activity of Aurora B persists from metaphase to the end of mitosis (2). Besides recent reports showing Aurora C to be a chromosomal passenger protein that binds directly to the inner centromere protein and survivin in vitro, not much is known about this member of the Aurora kinase family (6, 7).

Inappropriate levels of Aurora kinases are shown to be linked to genetic instability (8). Aurora kinases are overexpressed in a wide range of human cancers. Aurora A is commonly amplified in primary breast tumors, colorectal cancers, and in cancer cell lines of different origin including breast, ovarian, prostate, neuroblastoma, and cervix (9–11). Aurora B is also expressed at high levels in primary human colorectal cancers and other tumor cell lines (12). Therefore, Aurora kinases have become promising targets for cancer therapy. Considerable effort has been devoted in developing new small molecule inhibitors. ZM447439 (Astra Zeneca), Hesperadin (Boehringer Mannheim), VX-680 (Vertex Inc.), and AZD1152 are a few of the several small molecule inhibitors that have been evaluated (13, 14). The common cellular phenotype induced by inhibition of Aurora A kinase is failure of cytokinesis with the induction of polyploidy and multinucleated giant cells. This is in contrast to inhibition of Aurora B, which induces mitotic arrest followed by apoptosis. Interestingly, drugs that inhibit both Aurora kinases A and B induce multinucleated cells, indicating that the cellular phenotype associated with Aurora B inhibition is favored when both kinases are inhibited concurrently.
AZD1152 in Combination with CPT-11

Translational Relevance

Aurora B kinase inhibitors are now in clinical trials as single agents, and combinations with chemotherapy are planned. One such agent AZD1152 is active in vivo against colon cancer xenografts. We therefore elected to evaluate this drug with the topoisomerase I inhibitor CPT-11, in vivo, and its active metabolite SN-38, in vitro. As predicted, these drugs have distinct cell cycle effects such that the sequence of drug administration can either enhance or diminish the antitumor effect. These studies provide unique insights into the importance of examining drug sequence when administering an Aurora B kinase inhibitor with cytotoxic agents, especially those that affect the cell cycle. These results have promising clinical implications, as combinations of Aurora B kinase inhibitors with chemotherapy, including CPT-11, enter clinical trials.

Materials and Methods

Cell culture. The HCT-116 human colon carcinoma cell line was a generous gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Cultures were maintained at 37 °C in the presence of 5% CO₂ in McCoy's Media supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Colo-205, a colon carcinoma cell line, was a generous gift from Neal Rosen (Memorial Sloan Kettering Cancer Center, NY) and was maintained in RPMI media supplemented 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. AZD1152 and its active metabolite of AZD1152-HQPA were obtained from AstraZeneca. SN-38 was supplied by and CPT-11 was purchased from Pfizer Inc.

Clonogenic assay. Log-phase cells were plated, in triplicate, onto 100-mm dishes at 1,000/dish and were allowed to attach for 24 h before treatment. At the end of treatment, cells were cultured in drug-free media for 7 to 10 d. The resulting colonies were scored after staining with 0.01% crystal violet (19). Colorimetric cell proliferation assay. The assay was done as per the manufacturer's protocol (Dojindo Molecular Technologies, Inc.). Briefly, 3,000 cells were plated in 100 μl volume in each well of a 96-well plate and treatments were applied 24 h after plating. After the desired incubation period with the drug, 10 μl of CCK-8 solution were added to each well which were further incubated at 37 °C for 1 to 4 h. This assay quantifies the amount of formazan dye generated by the activity of dehydrogenases in the cells that is directly proportional to the number of living cells. Then the optical density at 450 nm to determine the cell viability was measured using Spectra Max 340 PC (Molecular Devices Corp.).

Apoptosis assay. Cells were treated with AZD1152-HQPA alone or in combination with SN-38 as indicated in each experiment, and FITC Annexin V (BD Pharmingen) was used to determine the percentage of cells within a population that were apoptotic as per the manufacturer's protocol. For combination studies, cells were treated with either agent alone for 24 h, drug was removed, floating cells were saved and returned to the plates, and drug in fresh media was added for an additional 24 h before assaying for apoptosis.

Quantitative fluorescent microscopy. Cells were collected as above after drug treatment and fixed in 3% paraformaldehyde. The nuclear morphology of cells was examined using fluorescence microscopy after staining with 4', 6-diamidino-2-phenylindole (DAPI) to a final concentration of 25 μg/mL. Cells were scored as apoptotic based on the presence of condensed fragmented chromatin. Multinucleated cells were defined as cells containing huge, multilobed nuclei. A minimum of 400 cells were counted for each sample and taken as a percentage of untreated cells.

Flow cytometry. Cell cycle analyses were done by harvesting both adherent and nonadherent cells. For flow cytometry, the cells were trypsinized, washed, and fixed in 75% ice cold ethanol. Cells were either stained with propidium iodide (50 μg/mL) containing RNase (5 μg/mL) for the measurement of DNA content or labeled with the phospho MPM-2 monoclonal antibody (Upstate), which recognizes specific phosphorylated epitopes present only during mitosis and subsequently with FITC-conjugated antimouse secondary antibody (ICN/Jackson Immuno Research) for the mitotic index measurement. Cells were then treated with RNase and propidium iodide. Samples were analyzed on a FACScan (Becton Dickinson) for cell cycle distribution and mitotic index (percentage of MPM-2–positive cells) using the Cell Quest software. For this analysis 40,000 events have been examined per sample.

Immunofluorescence assay. HCT-116 cells were plated in 2-well chamber slides and treated without or with varying concentrations of AZD1152, and cells were fixed in 4% paraformaldehyde for 10 min at room temperature. The cells were then blocked in 2% goat serum followed by incubation in rabbit polyclonal anti-phospho Ser 10

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histone H3 (Cell Signaling Technology; 1:250) and subsequently with Alexa Fluor-conjugated secondary antibody (Molecular Probes). DNA was labeled with DAPI at a final concentration of 25 μg/ml for 10 min.

Cell extraction, immunoprecipitation, and immunoblotting. Cell lysates were prepared as described by lysis of both floating and adherent cells in whole cell lysis buffer (50 mmol/L Tris, pH 8, 250 mmol/L NaCl, 0.5% NP-40, 0.2 mmol/L EDTA, 2 mmol/L EGTA, 10% Glycerol, 0.1 mmol/L phenylmethylsulfonylfluoride, 0.1 mmol/L Na3VO4, 1 mmol/L DTT, 1 μg/ml leupeptin, 2 μg/ml aprotenin, 1 μg/ml pepstatin), allowed to lyse on ice for 10 min, sonified, and cleared by centrifugation in a microcentrifuge at 13,000 rpm for 10 min at 4°C (20). Fifty micrograms of protein were fractionated by SDS-PAGE and transferred onto Immobilon membranes (Millipore). Equal protein loading was confirmed by Amido black staining (Bio-Rad). After blocking with 5% nonfat milk, membranes were probed with primary antibodies. The following antibodies were used in this study: mouse monoclonal to poly (ADP-ribose) polymerase (PARP) was from BD Biosciences; mouse monoclonal anti-p53 was from Santa Cruz Biotechnology; mouse monoclonal anti-p21 (1:1000) was from Oncogene; rabbit polyclonal to phosphorylated Ser 10 H3, rabbit polyclonal to cleaved caspase 3, and mouse monoclonal to cleaved PARP were from Cell Signaling; α-tubulin was from Upstate Biotechnology. Bound primary antibodies were detected with horseradish peroxidase–conjugated secondary antibodies (ICN/Jackson ImmunoResearch) and visualized by enhanced chemiluminescence reagent (Amersham Pharmacia).

Xenograft studies. Athymic mice bearing HCT-116 tumors (7 mice/cohort) of 200 mm3 diameter were treated with specified dose of AZD1152 (25 to 100 mg/kg/d) i.p. as a single agent, 2 d/wk for 3 wk. Twenty-four hours after each treatment on days 8 and 22, and 3 wk after the last treatment on day 43, one animal from each cohort was sacrificed and the tumors examined for H&E, Ki67 staining, and TUNEL. Tumors were measured every 2 to 3 d from each cohort) of 200 mm3 diameter were treated with specified dose of AZD1152 (25 to 100 mg/kg/d) i.p. as a single agent, 2 d/wk for 3 wk. Mice in the control groups were given vehicle (0.3 mol/L Tris at pH 9) alone. Twenty-four hours after the last treatment (day 22), two animals from each cohort were sacrificed and the tumors examined for H&E, Ki67 staining, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). For combination studies 7 mice/cohort received AZD1152 alone i.p. (100 mg/kg) daily ×2, CPT-11 alone i.p. (100 mg/kg, the maximum tolerated dose) ×1, or the same doses i.p. of AZD1152 on days 1 and 2 and CPT-11 on day 3 or CPT-11 on day 1 followed by AZD1152 for 2 d. Each treatment condition was repeated weekly for 3 wk. Mice in the control groups were given vehicle (0.3 mol/L Tris at pH9) alone. Twenty-four hours after the last treatment (day 22), two animals from each cohort were sacrificed and the tumors examined for H&E, Ki67 staining, and TUNEL. Tumors were measured every 2 to 3 d with calipers, and tumor volumes were calculated by the formula 4/3 x r3 (or decay), which are clearly violated for our data. P values were calculated using the Wilcoxon Rank Sum test.

Results

AZD 1152-HQPA induces growth arrest, apoptosis, and polyploidy in HCT116 cells. HCT-116 colon carcinoma cells were treated with varying concentrations of AZD1152-HQPA to determine its target specificity and its biological effects. As shown in Fig. 1A, AZD1152-HQPA, by both Western blot analysis and immunofluorescence, inhibited histone H3 phosphorylation, a known substrate of Aurora B, at concentrations of 20 nmol/L. As seen with DAPI staining, this corresponded to the induction of polyploid cells (cells with large, multilobed nuclei). Its effect on cellular proliferation was determined by clonogenic assay and the IC50 value was determined to be 20 nmol/L (Fig. 1B). However, there was no induction of apoptosis at this concentration. With higher concentrations (100-200 nmol/L), and with prolonged drug exposure (48 hours), induction of apoptosis could be detected by the appearance of a modest sub G1 peak using flow cytometry (Fig. 1C) and PARP cleavage by Western blot (Fig. 1D). AZD1152-HQPA showed no difference in the kinetics of mitotic entry or exit as compared with the untreated cells (not shown). However, cells treated with AZD1152-HQPA showed an increased DNA content from 2N to 4N to 8N despite the induction of p53 and p21 (Fig. 1C and D).

Single agent AZD1152 inhibits the growth of HCT-116 tumor xenografts. Because AZD1152-HQPA showed a very potent inhibition of proliferation in vitro, its efficacy was next tested in vivo with the prodrug AZD1152. Preliminary studies indicated that i.v. push for two days, i.p. injections for two days, and continuous infusion of AZD1152 for 48 hours using a mini-pump, gave identical results (data not shown). In view of this, all subsequent studies were conducted with i.p. injections. As shown in Fig. 2A, with 3 weekly treatments (arrows), even at the lowest dose of 25 mg/kg, tumor volume on day 23 was reduced to 50% when compared with untreated controls (P = 0.02). Tumor growth suppression was dose-dependent. Although tumor regression seemed to be more pronounced at 100 mg/kg, this was not significantly different from the 75 mg/kg results (P = 0.06). However, on day 23 the tumor regression for both the above doses was significantly greater than that observed with 50 mg/kg (P = 0.03). At all these doses there was no associated weight loss in the mice.

Tumors were collected 24 hours after the last treatment (day 23) and tested for the inhibition of Aurora B kinase activity by staining for phospho histone H3, inhibition of proliferation by Ki-67, induction of apoptosis by TUNEL, and...
changes in nuclear morphology by H&E. Figure 2B shows only 10% inhibition of Aurora B kinase activity with 25 mg/kg. However, there was 70%, 80%, and 90% inhibition of phospho-histone H3 at doses of 50, 75, and 100 mg/kg, respectively ($P = 0.03$; 50 mg/kg versus 75 and 100 mg/kg). Proliferation of tumor cells was similarly inhibited in a concentration-dependent manner, as determined by staining with Ki-67 ($P = 0.01$; 50 mg/kg versus 75 and 100 mg/kg; Fig. 2B). As shown in Fig. 2C (top, middle panel, arrow H&E staining), the cells were polyploid with enlarged nuclei, presumably due to the inhibition of cytokinesis and continued endoreduplication, a known effect of Aurora B inhibition. This was associated with decreased Ki-67 staining (Fig. 2C, bottom, middle panel), indicating a significant decrease in cellular proliferation. There was minimal induction of apoptosis by TUNEL under any condition tested (data not shown), suggesting that the decrease in tumor volume (Fig. 2A) with single agent AZD1152 was related to inhibition of cellular proliferation.

In order to determine whether the growth inhibition was reversible, the tumor volume was monitored for 3 weeks after the last drug administration. At the end of 3 weeks (day 44), tumor volumes had increased back to baseline (data not shown). Tumors were then collected and stained for Ki-67, H&E, and TUNEL. As shown in Fig. 2C (right panel), Ki-67 staining showed an increased proliferation rate. H&E staining showed a restoration of mononuclear cells with a decrease in the giant multinucleated cell population that had been observed at the end of treatment (day 23). These results suggest that inhibition of tumor growth by an Aurora B kinase inhibitor requires sustained induction of the polyploid phenotype in order to maintain the decrease in cellular proliferation that is associated with inhibition of tumor growth. Interestingly, when rechallenged with AZD1152, a decrease in tumor growth was observed in these animals, indicating that the tumors did not acquire resistance to the effects of Aurora B inhibition (data not shown).

SN-38 in combination with AZD1152-HQPA. CPT-11 is a topoisomerase I poison and is used as a standard part of chemotherapy for the treatment of metastatic colon cancer (23). Because our long-term goal is to combine AZD1152 with cytotoxic chemotherapy for the treatment of colon cancer, we elected to test AZD1152-HQPA (the active agent) in vitro with SN-38, the active metabolite of CPT-11, against the HCT-116 cells. As these drugs have independent effects on the cell cycle, we elected to test these drug combinations in a sequence-specific manner. The combination was first tested for its effect on clonogenicity and cell viability. SN-38 alone has been shown to have a profound effect on inhibition of clonogenicity, even at concentrations as low as 1 nmol/L, due to induction of p53 and p21 and a resulting G2 arrest (22). In view of this, differences in clonogenicity are generally difficult to show with SN-38 in combination therapy. Nevertheless, as shown in Fig. 3A, the inhibition of clonogenicity favored AZD->SN (P = 0.0001, AZD->SN versus SN->AZD, and all other comparisons showed a $P < 0.005$). Even a modest effect was observed with AZD->SN over AZD+SN ($P = 0.0007$). This could be due to the escape of cells from G2 arrest, induced by SN-38 at the concentration used (1 nmol/L). Because multiple comparisons have been applied for AZD->SN, the Bonferroni adjustment was applied and all comparisons were shown to be statistically significant. However, inhibition of cell viability
using Dojindo cell proliferation kit, was significantly greater for AZD->SN than AZD, SN, or SN>AZD (P > 0.001, 0.002, and 0.01) and for any of the combinations tested, including AZD+SN (P = 0.03). In this assay, after Bonferroni correction, all statistical comparisons were statistically significant except for the concurrent treatment, which, though individually significant, fell on the borderline of 0.03. This same sequence dependency was observed in the Colo-205 cells, such that AZD->SN had the greatest effect on inhibiting cell growth (Supplement 1A).

We next examined the effects of the combinations on cell cycle distribution by flow cytometry. As predicted (Fig. 3B), SN-38 alone (SN) at a pharmacologically relevant concentration (20 nmol/L) induced a permanent G2 (4N DNA) arrest. This effect was sustained for at least another 24 hours even when these cells were placed in drug-free media (22). This was in contrast to AZD1152-HQPA (AZD, 100 nmol/L) which induced endoreduplication, resulting in polyploidy (8N DNA).

However, when SN-38 was given prior to (SN->AZD) or concomitantly with AZD1152-HQPA (AZD+SN), SN-38 blocked induction of polyploidy by AZD1152-HQPA. This was due to the induction of permanent G2 arrest by SN-38, thereby inhibiting cells from entering mitosis where AZD1152-HQPA is active. A small 8N peak was observed with AZD+SN, suggesting those cells that were out of G2 and were first affected by AZD1152-HQPA. On the other hand, when AZD1152-HQPA was given prior to SN-38 (AZD->SN), the 8N peak was comparable with AZD alone, and there was a large increase in the sub G1 (<2N) population, reflecting an enhancement of apoptosis (15 ± 1% with AZD alone to 30 ± 3% with AZD->SN). Of particular note, with SN->AZD a significant reduction in the sub G1 peak was observed (8 ± 1%), compared with AZD alone (P < 0.01).

The induction of apoptosis by SN-38 and AZD1152-HQPA is sequence-specific. In order to further understand the mechanism for these sequence-dependent effects, we next elected to

![Fig. 2. AZD1152 induces tumor regression of HCT-116 xenografts. A, athymic mice were implanted with HCT-116 tumors. The mice (n = 7) were treated with 25, 50, 75, and 100 mg/kg of AZD1152 or vehicle i.p. daily for two consecutive days each week for 3 wk. Arrows, weekly treatment. Tumor volume was measured every 2 to 3 d and mean tumor volume was plotted against time in days. B, 24 h after the final treatment (day 23) tumors were excised and quantified by immunohistochemistry for percent reduction in phospho H3 and Ki-67 staining at each treatment dose compared with control. C, H&E staining shows polyploid (multilobed nuclei, arrow heads) at the end of treatment (day 23) and disappearance of the giant nuclei 3 wk later (day 44). A decrease (day 23) in Ki-67 followed by an increase (day 44) was observed. A representative experiment is shown.](https://www.aacrjournals.org/doi/10.1158/1078-0432.CCR-09-0648)
examine this further by quantitative fluorescent microscopy (DAPI) and Annexin V labeling in the HCT-116 cells. As shown in Fig. 4A, examination of the DAPI-stained nuclear morphology by fluorescence microscopy showed enlarged interphase nuclei with the SN, SN->AZD, and the SN+AZD combinations. In contrast, AZD treatment alone resulted in large multilobed nuclei, whereas AZD->SN showed decondensed apoptotic nuclei (arrow heads), besides the multilobed nuclei, indicating both polyploidy and apoptosis. The induction of apoptosis increased from 15 ± 2% with AZD alone to 29 ± 4% with AZD->SN (P = 0.013), an effect that was quite similar to that observed with analysis of the sub G1 peak. Annexin V staining (Fig. 4B), which detects both early and late stages of apoptosis, further confirmed the increase in apoptosis with the AZD->SN combination, when compared with the other conditions tested. Again, using both quantitative fluorescent microscopy and Annexin V, the reverse sequence of SN->AZD showed a partial reversal in the induction of apoptosis induced by AZD alone. In addition, Western blot analysis also showed a large increase in PARP cleavage with AZD->SN, which is not observed with the other combinations (Fig. 4C). As determined by quantitative fluorescent microscopy, sub-G1 peak, and PARP cleavage, the effect of enhanced apoptosis with the sequential AZD->SN was also observed in Colo-205 cells [Supplement 1B to D]. Taken together, these data indicate that cells which have undergone AZD1152-HQPA–induced endoreduplication are susceptible to apoptosis with subsequent SN-38 therapy. In contrast, SN-38 can induce a prolonged G2 arrest, which is sufficient to prevent the induction of both polyploidy and apoptosis by subsequent AZD1152-HQPA therapy. This finding also strongly indicates that the sequence of drug administration is critical to achieve the full combination effect.

**CPT-11 enhances the effect of AZD1152 in vivo.** Based on the increased efficacy of the AZD->SN-38 sequence combination, we chose to compare AZD1152 in sequence with CPT-11

**Fig. 3.** SN-38 (SN) antagonizes or enhances the effect of AZD1152-HQPA (AZD) in a sequence specific manner. A, clonogenicity (SN 1 nmol/L, AZD 15 nmol/L) and cell mass (SN 50 nmol/L, AZD 100 nmol/L), as determined by the Dojindo Cell Counting Kit (see Materials and Methods), indicating the % of growth/proliferation inhibition as compared with control. B, (i) flow cytometric analysis showing a G2 (4N) arrest upon SN treatment and induction polyploidy (8N) in AZD-treated HCT-116 cells. The effect of SN predominates (4N) when SN is given before or concurrently with AZD, but enhanced cell death (<2N, sub G1 peak) and maintained polyploidy (8N) is observed when AZD is given before SN. (ii) The percentages of cells in sub G1 for each condition is shown. Experiments were carried out in triplicate or, sextuplicate and were repeated thrice. Error bars, 1 SD.

**Fig. 4.** AZD->SN induces apoptosis in HCT-116 cells. A, HCT-116 cells were treated with SN-38 (SN) or AZD1152-HQPA (AZD) alone or in combination and checked for nuclear morphology after DAPI staining. Enlarged interphase nuclei are shown with SN->AZD, SN+AZD, and SN alone (bottom panel); polyploid, multilobed nuclei with AZD alone; and multilobed nuclei with decondensed apoptotic nuclei (arrows) with AZD->SN treatment. B, percentage of HCT-116 cells undergoing apoptosis by quantitative fluorescent microscopy and Annexin V staining. C, Western blot analysis for PARP cleavage of HCT-116 cell lysates after treatment with AZD and SN combinations. α-Tubulin is shown to confirm equal loading of protein. Experiments were repeated at least three times. Error bars, 1 SD.
with the other treatment conditions in HCT-116 xenografts. As AZD1152 at 100 mg/kg induced at least 90% inhibition of phospho-histone H3 without weight loss, we selected this dose for combination therapy. All animals were treated on days 7, 14, and 21. As shown in Fig. 5A, all treatment conditions produced comparable levels of growth suppression, but the time of onset of tumor regrowth and the percent increases in tumor volume after completion of therapy were different between groups. Tumor regrowth was noted as early as day 29 for CPT-11 alone, day 36 for AZD1152 alone, day 43 for CPT-11->AZD, and day 50 for AZD->CPT-11. In terms of overall increases in tumor volume, both sequential combinations at day 60 showed greater inhibition of tumor regrowth when compared with either AZD1152 alone (P = 0.06, AZD versus CPT-11->AZD; P = 0.008, AZD versus AZD->CPT-11) or CPT-11 alone (P = 0.008, CPT-11 versus CPT-11->AZD; P = 0.008, CPT-11 versus AZD->CPT-11). Furthermore, a comparison of the two sequential combinations indicates that the overall increase in tumor volume up to day 71 was significantly less for AZD->CPT-11 than for CPT-11->AZD (P = 0.02). There was no associated weight loss with any condition tested.

These data suggest that AZD1152 followed by CPT-11 is superior to CPT-11 followed by AZD1152, but the differences between the sequential combinations were not as dramatic as expected from the in vitro studies, in which polyploidy induced by the Aurora B kinase inhibitor was essentially inhibited when SN-38 was given before AZD1152-HQPA. This could be due to the less effective in vivo conversion of a prodrug to its active metabolite, but a more likely explanation could be due to cell cycle interactions between the two agents. Therefore, the tumor samples were evaluated by H&E for morphology, Ki-67 for proliferation, and TUNEL for apoptosis 24 hours after the last treatment. As shown in Fig. 5C, AZD1152 alone again induced large numbers of giant, multinucleated cells. However, with AZD->CPT-11 there were fewer appreciable tumor cells present, and those that were present were bizarrely shaped (right, top panel). In addition, when compared with the other treatment conditions, apoptosis by TUNEL increased from 19 ± 5% with AZD alone to 48 ± 5% with AZD->CPT-11 (Fig. 5C, P = 0.036).
Nevertheless, with Ki-67 staining, some cells continued to proliferate, suggesting an explanation for the modest degree of tumor regrowth. Interestingly, Ki-67–positive cells could still be detected after CPT-11 and CPT->AZD treatment, indicating that cells had escaped from G2 arrest, and continued to proliferate. Therefore, it seems that even at the maximum tolerated dose of CPT-11 (100 mg/kg), some tumor cells are still able to escape from G2 cell cycle arrest, making them susceptible to the subsequent effect of AZD1152 therapy. This would explain the increase in apoptosis observed with sequential CPT->AZD as compared with CPT alone (Fig. 5C). Nevertheless, CPT-11 before AZD1152 still seemed to partially inhibit the induction of apoptosis observed with AZD alone and was inferior to AZD->CPT (Fig. 5C). Collectively, these results are consistent with what was observed in vitro with the combination therapies (Fig. 4).

Discussion

AZD1152 is a phosphate prodrug that is rapidly converted in plasma to the active species AZD1152-HQPA (14). AZD1152-HQPA is a selective inhibitor of Aurora B kinase with an IC50 value in the low nanomolar range against a range of solid tumor cell lines (14, 16, 17). Consistent with these findings, AZD1152-HQPA showed potent inhibition of proliferation of HCT-116 cells with an IC50 value of 20 nmol/L. The target inhibition was confirmed by examining Histone H3 phosphorylation (Ser10), a known substrate of Aurora B kinase. As shown previously (14), AZD1152-HQPA induced significant polyploidy (8N DNA) with large multilobed nuclei. Apoptosis was induced in a dose-dependent manner and upon prolonged exposure of the drug. The sensitivity to growth inhibition in vitro was confirmed in vivo in mice bearing HCT-116 tumor xenografts. In these in vivo experiments, a dose-dependent inhibition of tumor growth was observed with significant tumor regression at higher but well-tolerated doses (75-100 mg/kg). This was associated with inhibition of Histone H3 phosphorylation (Ser10) and induction of multinucleated cells with decreased cellular proliferation but with little evidence for apoptosis as indicated by TUNEL staining.

The lack of single-agent activity observed with AZD1152, as well as with other Aurora B kinase inhibitors, in solid tumors currently in phase I clinical trials may be due to its inability to induce apoptosis (24–26). In view of this, we elected to explore whether SN-38 in vitro and CPT-11 in vivo could enhance the effect of AZD1152. Because SN-38 induces DNA strand breaks following by a permanent G2 arrest (22), we elected to determine whether SN-38 would block the mitotic effect of AZD1152-HQPA. The combination of SN-38 with AZD1152-HQPA showed interesting and promising results that were sequence-dependent. As expected, when SN-38 was given prior to or concomitantly with AZD1152-HQPA, the cells were arrested in G2, blocking cells from entering mitosis. AZD1152-HQPA is active. This prevented both AZD1152-HQPA–induced polyploidy and apoptosis observed with AZD1152-HQPA alone. However, when AZD1152-HQPA was given prior to SN-38, the effect of AZD1152-HQPA in inducing polyploidy was sustained and SN-38 seemed to enhance the induction of apoptosis on the endoreduplicating cells.

Previous studies have shown that single agent AZD1152-HQPA can induce apoptosis with higher drug concentrations and with protracted exposure, but these results indicate that a greater degree of apoptosis may be possible when the drug is combined with an inhibitor of topoisomerase I. This was confirmed in mice bearing HCT-116 xenografts, such that both sequential combinations seemed more potent than either single agent therapy in inhibiting tumor regrowth. Upon examining the tumor samples following CPT-11 followed by AZD therapy, it was evident that unlike the in vitro studies with 20 nmol/L SN-38, not all the cells were arrested in G2 after CPT-11. Therefore, those cells that escaped the arrest induced by CPT-11 were still susceptible to the effect of AZD1152. Nevertheless, when AZD1152 was administered first (AZD->CPT), cells were able to undergo endoreduplication, thus providing greater numbers of cells to be affected by subsequent CPT-11 treatment, resulting in an enhancement of apoptosis and a greater in vivo treatment effect.

Taken together, our findings strongly indicate that CPT-11 can enhance the effect of AZD1152 without inducing unwanted toxicities. They confirm the potential impact and utility of specific drug sequencing, particularly when individual drugs (even conventional cytotoxic agents) have specific cell cycle effects. In fact, use of the wrong sequence of administration result in a process termed “cell-cycle mediated drug resistance,” such that the cell cycle interactions can result in antagonism (27). Similar effects have been reported with other cell cycle inhibitors when combined with chemotherapy (28). In the current studies, the in vivo effects did not exactly mirror the in vitro effects. This finding does suggest that the most efficient sequence of drug administration would be AZD1152 followed by CPT-11. Based on these results, sequential combinations of AZD1152 followed by CPT-11 can now be considered.

Disclosure of Potential Conflicts of Interest

G. Schwartz, advisory board, AstraZeneca.

References


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