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**Title:**

Inhibiting Wee1 and ATR Kinases Produces Tumor-Selective Synthetic Lethality and Suppresses Metastasis

**Authors and Affiliations:**

Amirali B. Bukhari¹, Cody W. Lewis¹, Joanna Pearce¹, Deandra Luong¹, Gordon K. Chan¹, Armin M. Gamper¹*

¹ Cross Cancer Institute, Department of Oncology, University of Alberta, Edmonton, Canada

**Running Title:** Targeting Wee1 and ATR Inhibits Tumor Growth and Metastasis

**Key words:** DNA damage response; synthetic lethality; metastasis; cancer stem cells; kinase inhibitors; breast cancer

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*Corresponding Author:*

Armin Gamper
Department of Oncology
University of Alberta
Cross Cancer Institute
11560 University Avenue
Edmonton, Alberta T6G 1Z2
Tel.: 780-432-8436
Fax: 780-432-8892
Email: gamper@ualberta.ca

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Abstract

We used the cancer intrinsic property of oncogene-induced DNA damage as the base for a conditional synthetic lethality approach. To target mechanisms important for cancer cell adaptation to genotoxic stress and thereby to achieve cancer cell-specific killing, we combined inhibition of the kinases ATR and Wee1. Wee1 regulates cell cycle progression, whereas ATR is an apical kinase in the DNA damage response. In an orthotopic breast cancer model, tumor-selective synthetic lethality between bioavailable ATR and Wee1 inhibitors led to tumor remission and inhibited metastasis with minimal side effects. ATR and Wee1 inhibition had a higher synergistic effect in cancer stem cells than in bulk cancer cells, compensating for the lower sensitivity of cancer stem cells to the individual drugs. Mechanistically, the combination treatment caused cells with unrepaired or under-replicated DNA to enter mitosis leading to mitotic catastrophe. As these inhibitors of ATR and Wee1 are already in phase I/II clinical trials, this knowledge could soon be translated into the clinic, especially as we showed that the combination treatment targets a wide range of tumor cells. Particularly the anti-metastatic effect of combined Wee1/ATR inhibition and the low toxicity of ATR inhibitors compared to Chk1 inhibitors has great clinical potential.
**Introduction**

The DNA Damage Response (DDR) senses DNA damage and replication stress and orchestrates the cellular response to protect the cell and organism from genotoxic insults. These signal transduction pathways include the choreography of DNA repair, cell cycle control, and cell fate decision among others (1). Due to their dysregulated proliferation, the genomic integrity of cancer cells is particularly threatened by DNA damage and replication stress, but also by metabolic, mitotic, oxidative and proteotoxic stresses (2). Furthermore, during tumorigenesis cells often lose DDR mechanisms leading to increased genomic instability (3). These DNA repair/DNA damage signaling defects and/or the increased genotoxic stress make cancer cells heavily dependent on the (remaining) intact DDR pathways.

Synthetic lethality refers to an interaction between two genes when the perturbation of either gene alone is viable but the simultaneous perturbation of both genes leads to cell death. The discovery that breast cancer cells with mutations in homologous recombination proteins BRCA1 or BRCA2 are hypersensitive to poly (ADP-ribose) polymerase (PARP) inhibitors (4, 5) led to therapeutic approaches targeting cancer cells with deficiencies in one DDR pathway by inhibition of an alternative DDR pathway. However, as this approach only targets cells with a defective DDR, it is bound to only affect a subset of cancers or populations within a tumor. Resistance can arise by reactivation of the defective pathway. Conditional synthetic lethality refers to synthetic lethality observed only under certain circumstances, such as genetic background or metabolic state of cells or cellular environment (6). In view of the inter- and intratumor heterogeneity commonly observed, to achieve the necessary therapeutic window for a wide range of tumors it is necessary to identify a common “condition” upon which to base cancer-selective conditional synthetic lethality. Oncogene-induced DNA damage is a common feature of cancer cells leading to high levels of
replication stress as well as mitotic stress in cancer cells compared to normal proliferating cells (7, 8). Based on this tumor-specific property, we tested whether increasing genotoxic stress and simultaneously inhibiting an important rescue pathway would lead to cancer cell-selective death by evaluating the efficacy of combined ATR and Wee1 inhibition on cancer cell eradication. Wee1 is a kinase controlling G/M and S phase checkpoints via phosphorylation of the cyclin dependent kinases CDK1 and CDK2. Furthermore Wee1 inhibition prolongs mitosis in a range of cancer cells and makes them more susceptible to chemotherapy-induced mitotic catastrophe (9). Ataxia telangiectasia and Rad3 related (ATR) is the apical kinase of a DDR pathway. ATR is considered the main mediator in the DDR to replication stress (7), including signaling to cell cycle checkpoints via Chk1 and regulating repair by promoting extensive DNA end-resection needed for homologous recombination (10-12). Bioavailable selective ATR inhibitors (AZD6738 by AstraZeneca; VX-970 and VX-803 by Merck) as well as the Wee1 inhibitor AZD1775 have recently entered phase I/II clinical trials in combination with radiation or chemotherapeutics.

Here we report that tumor-selective synthetic lethality between ATR and Wee1 inhibitors leads to tumor shrinkage and suppresses metastasis. Using an orthotopic breast cancer xenograft mouse model we show that combination treatment leads to complete remission in 6/9 cases, inhibits tumor spread and prolongs survival. Our toxicity studies show that the combination treatment is associated with minimal side effects. Fast proliferating tissues, such as the ileum or the bone marrow, showed no signs of renewal defects. Synergistic cell killing by inhibition of ATR and Wee1 is observed in cancer cells from various tissue origins, but not in untransformed cells. Mechanistic studies using pulses of reversible inhibition during the cell cycle show that combined ATR/Wee1 inhibition during S and G2/M phase cooperate to kill cancer cells. Furthermore, live cell imaging studies reveal that combined ATR/Wee1 inhibition causes cells to enter mitosis with
unrepaired/under-replicated DNA leading to mitotic catastrophe. As the studied ATR and Wee1 inhibitors are already in phase I/II clinical trials, this knowledge could soon be translated into the clinic.
Results

Synergistic cell killing of cancer cells by ATR and Wee1 inhibition in vitro

CDK1/2 activity is regulated by inhibitory phosphorylation at tyrosine 15 by the protein kinase Wee1 that is counteracted by the phosphatase cdc25. CDK1 activity regulates entry into and exit out of mitosis (13-17), and we recently showed that Wee1 inhibition in breast cancer cells promotes premature mitosis, prolongs mitosis, and promotes paclitaxel-induced mitotic catastrophe (9). In addition to regulating entry into mitosis, screens identified an important role for Wee1 in the maintenance of genome integrity during DNA replication. Both Wee1 knockdown or inhibition lead to upregulation of phosphorylated H2AX (γH2AX), a readout for DNA damage, in S phase cells (18-20). The underlying mechanisms remain poorly understood and seemingly conflicting data led to two models proposing either that Wee1 controls genomic stability during replication by regulating origin firing (19), or that it regulates the processing of stalled replication forks by the Mus81-Eme1 endonuclease (20).

The protein kinase ATR is constitutively bound by ATRIP (ATR-interacting protein) and is activated by replication protein A (RPA)-coated single-stranded DNA, structures that can arise from stalled replication forks or resected DNA double-strand breaks (1). Unsurprisingly, ATR plays a crucial role in the response to replication stress – likely the reason for it being an essential gene (21, 22) – and to ionizing radiation-induced DNA double-strand breaks. ATR activation is important for S and G2/M checkpoint signaling and DNA damage repair by homologous recombination (10).

To test whether Wee1 inhibition activates ATR, we incubated cancer cells for 2 hours with the Wee1 inhibitor AZD1775. Immunoblots of cell lysates show that AZD1775 treatment leads to phosphorylation of Chk1 Serine 345, a target site of ATR (Figure 1A). ATR activation was
confirmed by co-treatment with two ATR selective inhibitors, AZD6738 and ETP-46464, which suppressed AZD1775-induced Chk1 phosphorylation (**Figure 1A, lanes 1-4**), and is observed in breast cancer (MDA-MB-231) and osteosarcoma (U-2 OS) cells, indicating that it is unlikely cancer type specific (**Figure 1A, Supplementary Figure 1**). ATR activation by AZD1775 is potentiated by DNA damaging agents, such as ionizing radiation (**Supplementary Figure 1**). The activation of ATR by Wee1 inhibition prompted us to study the combinatorial effect of Wee1 and ATR inhibition on cancer cell killing. 5,000 cells were plated and incubated with different concentrations of AZD1775 and AZD6738 for 4 days before measuring surviving cells by Crystal Violet staining and colorimetry (23). We observe **synergistic** cell killing by ATR and Wee1 inhibition in all tested cancer cell lines (**Table 1, Supplementary Figure 2C**), including the human breast cancer cell lines MDA-MB-231, MCF7, and Zr-75-1 (**Figure 1B-D**), but not in non-tumorigenic MCF 10A and immortalized mammary epithelial cells (hTERT-HME1) (**Figure 1E, F**), as demonstrated in Loewe plots and calculated Bliss combination indices (CI) (24). A CI below 1 indicates synergy. The synergistic cell killing we observe with Wee1 and ATR inhibitors is unlikely due to off-target effects, because several ATR inhibitors (including ETP-46464 and VE821, **Supplementary Figure 3**) and knockdown of Wee1 with siRNA (**Supplementary Figure 2D**) show cooperative lethality as well. Importantly, and in agreement with a conditional synthetic lethality of Wee1 and ATR based on DNA damage, a favorable therapeutic window for the combination treatment is provided by the increased oncogenic stress in cancer cells, as no cooperative lethality is observed in MCF 10A and hTERT-HME1. This is in stark contrast to inhibition of the ATR downstream target Chk1. MCF 10A and hTERT-HME1 are very sensitive to the Chk1 inhibitor UCN-01 alone and to combined Wee1 and Chk1 inhibition (**Supplementary Figure 4**). Depletion or inhibition of Chk1, but not of ATR, has previously been shown to cause
DNA damage in normal cells (25), likely explaining the toxicity of Chk1 inhibitors observed in the clinic (26, 27). Several studies have shown that in the absence of exogenous genotoxic stress ATR inhibitors are well tolerated (28, 29) and cells from Seckel syndrome patients, who have hypomorphic levels of ATR, do not show increased DNA damage levels (30), indicating that low ATR activity is sufficient to respond to the endogenous genotoxic stress in normal cells.

**Combination treatment of cancer cells with ATR and Wee1 inhibitors leads to centromere fragmentation and mitotic catastrophe**

Several reproductive cell death modes can lead to the inability of a cell to reproduce after exposure to genotoxic stress (31, 32): While treated cells with intact cell cycle checkpoint function tend to senesce, the major death mechanism after exposure to DNA damaging agents for cells with defects in cell cycle checkpoints and impaired DNA repair mechanisms is *mitotic catastrophe*. Mitotic catastrophe occurs when cells enter mitosis prematurely before the completion of DNA repair and/or DNA replication, resulting in dysregulated/failed mitosis, and can lead to delayed apoptosis, senescence or even necrosis.

We used live cell microscopy to address whether cell death by Wee1 and/or ATR inhibition requires cells to enter mitosis. Cancer cell lines display variable intra-line (within their population) response to drug treatments (33). Therefore, monitoring individual cell fates with time-lapse microscopy is essential to understanding the cell cycle response of cancer cells to drug treatment. Breast cancer cell lines stably expressing GFP-tubulin and mCherry-histone H2B enabled us to track the fates of individual cells and their progenies. Our data for MDA-MB-231 show that, unlike Wee1 inhibition (*P* = 0.0387, one-way ANOVA) (9), ATR inhibition alone does not prolong mitosis (**Figure 2A, B**). Yet when ATR and Wee1 inhibition are combined, mitosis is significantly longer (*P* < 0.0001, one-way ANOVA) (**Figure 2A, B**) and commonly leads to cell death (**Figure**
The median time between nuclear envelope breakdown to anaphase in control cells, cells treated with AZD6738, AZD1775, or the combination is 35, 45, 160, or 325 minutes, respectively (Figure 2B). Cell death is observed during failed mitosis, after mitotic slippage (when cells have aborted mitosis as evidenced by the disappearance of the mitotic spindle without cytokinesis), or in interphase after cytokinesis (often with visible micronucleation) (Figure 2C, D; Supplementary Figure 5A). Mitotic duration seems to correlate with cell death observed during mitosis, with 0, 3.6, 28.6, or 64.3 percent of MDA-MB-231 cells dying in mitosis when treated with vehicle, AZD6738, AZD1775, or combined AZD6738/AZD1775, respectively (Figure 2D).

While ATR inhibition kills 44.6% of the cells, most of the cell deaths occur during interphase in daughter cells. We do not observe interphase death in cells before aborted or completed mitosis. This clearly indicates the importance of cells entering mitosis, presumably with unrepaired or under-replicated DNA, for cell death and shows that mitotic defects can lead to delayed cell death in daughter cells.

Mitotic cells with under-replicated genome (MUG) were discovered 30 years ago (34). Mitotic defects observed in these cells commonly include centromere fragmentation (35), characterized by the formation of centromere clusters spatially separated from the main mass of chromosomes. As the majority of cells treated with combined ATR and Wee1 inhibitors died in mitosis, we synchronized cells in S phase by a double thymidine block and inhibited ATR and/or Wee1 after release. Four hours after G1/S release, cells were fixed and stained for tubulin, centromeres and DNA (Figure 2E). Wee1 inhibition, but particularly combined ATR/Wee1 inhibition, leads to an increase in mitotic cells (Figure 2F) in the breast cancer cell lines MDA-MB-231 and T-47D, as well as in HeLa cells (Supplementary Figure 5B). Furthermore, the majority of the mitotic cells in the combination treatment group show centromere fragmentation, as seen by the clustering of
centromeres and kinetochores and their separation form the bulk condensed chromatin (compare mitotic cells treated with combined AZD6738 and AZD1775 to DMSO control in Figure 2E, Supplementary Figure 5B).

**Events in S phase and G2/M phase contribute to the synergistic cancer cell killing by the combination treatment of cancer cells with ATR and Wee1 inhibitors**

To estimate the contribution of abrogation of cell cycle checkpoints and DNA damage repair to overall cell killing, we evaluated the impact of ATR and/or Wee1 activity during phases of the cell cycle on cancer cell survival. As this requires the ability to switch ATR and Wee1 activity on and off, we tested the reversible nature of inhibition by AZD6738 and AZD1775. Mock or AZD6738 treated cells were UV-irradiated and ATR activation measured by Chk1 phosphorylation (Figure 3A). AZD6738 washout leads to ATR reactivation within 1 h, as evidenced by restoration of high Chk1 pS345 levels. AZD1775 treatment of cells reduces CDK1 phospho-Y15 levels, confirming that Wee1 is the primary kinase phosphorylating CDK1 at tyrosine 15 (Figure 3B). Washout of AZD1775 restores Wee1 kinase activity to full levels in less than two hours, as shown by the reestablishment of normal CDK1 phospho-Y15 levels. Having established that ATR and Wee1 inhibition can be reversed within approximately one hour, we next synchronized U-2 OS cells by a thymidine-nocodazole block (Figure 3C) as described (10). At various times after nocodazole release and for different durations, cells were pulse-treated with 1 μM AZD6739 and/or 300 nM AZD1775 by addition and subsequent washout as indicated: from +10 to +16 h (roughly late G1 to G2), from +18 to +22 h (late G2 into mitosis), from +10 to +22 h, for a full cell cycle starting from late G1, or for the entire period of 4 days. All cells were assayed for survival after 4 days by measuring Crystal Violet staining compared to mock treated cells. As discussed previously, treatment for the entire time window with a combination of AZD6738 and AZD1775 leads to
strong synergy (Figure 3D, right panel). Inhibition of ATR or Wee1 alone for short intervals, during S phase or late G2/mitosis (Figure 3D, first two left panels), had no significant effect on survival, indicating that cells were able to recover from transient ATR or Wee1 inhibition for the indicated time intervals. Prolonged inhibition, from late G1 into mitosis, on the other hand, leads to significant cell killing by the single agents ($P < 0.0001$, one-way ANOVA), comparable to inhibition for an entire cell cycle. Interestingly, combined ATR and Wee1 inhibition for just the short periods encompassing S phase (+10 to +16 h) or from late G2 into mitosis (+18 to +22 h) leads to killing of approximately half of the cells ($P < 0.0001$, one-way ANOVA). Yet when ATR and Wee1 are both inhibited from late G1 into mitosis (+10 to +22 h), less than 10% of the cells survive, indicating not only a strong synergy between the two inhibitors, but also the contribution of events during both cell cycle intervals (G1 to G2; G2 and mitosis) the inhibitors were active (compare the three left panels in Figure 3D). Combination treatment for an entire normal cell cycle interval further increased cell killing to levels comparable to treatment for the entire 4 days.

We also tested inhibitor-induced changes in cell cycle profiles in cells synchronized by a thymidine-nocodazole block, if AZD6738 and/or AZD1775 were added to G1 cells 6 hours after release. Flow cytometry of propidium iodide stained cells show a significant increase of cells with DNA content between 2n and 4n at 14 hours after nocodazole release in the combined ATR and Wee1 inhibitor treated group compared to control (Figure 3C). The DNA content indicates delayed S phase or entry into G2/mitosis with under-replicated genomes. The latter is more likely, because many cells retain a DNA content below 4n even several hours later. Combined with our observation that cells treated with both ATR and Wee1 inhibitor show frequent centromere fragmentation in mitosis (Figure 2E, F), a hallmark of under-replicated cells entering mitosis, the
inhibitor-induced shift in DNA content profile underlines the synergistic contribution of reversible ATR/Wee1 inhibition during S and G2/M phases in causing mitotic catastrophe.

**Combined inhibition of ATR and Wee1 leads to increased DNA damage in tumors in vivo**

AZD1775 and AZD6738 are both bioavailable and can be administered to mice by oral gavage. To test synthetic lethality between Wee1 and ATR inhibitors in tumors, we established a human breast cancer xenograft model in mice. Due to the tumor (micro)environment, drugs that sensitize in vitro face additional challenges in selectively killing cancer cells in vivo. The different growth kinetics in vivo, hypoxia, intra-tumoral heterogeneity, interaction with the stroma, and of course drug delivery, influence efficacy. Moreover, side effects such as injury to normal tissues are of great concern.

We derived from MDA-MB-231, a triple negative human breast cancer cell line (p53 mutated, BRCA wild type), a cell line that expresses the second-generation, less immunogenic firefly luciferase and the red-fluorescent protein tdTomato (36) ([Supplementary Figure 12](#)). In our orthotopic xenograft model these MDA-MB-231-\textit{fluc2-tdTomato} cells are injected into the fourth mammary fat pad of 6-8 week old female NOD.Cg-\textit{Prkd\textsuperscript{cscid}} \textit{Il2rg\textsuperscript{tm1Wjl}/SzJ} (NSG) mice according to our approved animal protocol (AC16225). Once tumors reach a volume of 40-50 mm\textsuperscript{3} they are randomly allocated to treatment or vehicle arms. For our initial biomarker study to validate in vivo inhibition of ATR and Wee1 by our inhibitors and to test DNA damage induction in tumors, we administered 25 mg/kg AZD6738 and 60 mg/kg AZD1775 by oral gavage daily over 5 days. One hour after the last drug treatment [the approximate T\textsubscript{max}, when these drugs show maximal plasma concentrations (37), (personal communication by AstraZeneca)] we harvested the tumors. Excised tumors (n = 3 mice per treatment group) were tested for ATR and Wee1 activity by immunohistochemistry, assessing phosphorylation of the respective Wee1 and ATR substrates.
CDK1 Y15 and ATR T1989 (Figure 4A, B) [As all Chk1 pS345 antibodies we tested did not work for immunohistochemistry, we used ATR auto-phosphorylation on Thr1989 as alternative readout for ATR activation (38) (See Supplementary Figure 6)]. Interestingly we not only confirmed ATR and Wee1 inhibition by AZD6738 and AZD1775, respectively, but also observed ATR activation in vivo in Wee1 inhibitor-treated tumors (Figure 4A). Our data also indicate that ATR or Wee1 inhibition over the same period leads to a significant increase in tumor cells with DNA damage, assayed by γH2AX staining (Figure 4C, D). Of note, combination treatment with the two kinase inhibitors seems to synergistically enhance the number of cells staining for γH2AX in the tumor (P < 0.0001, one-way ANOVA) (Figure 4D). It also reduces the fraction of proliferating tumor cells, as measured by Ki67 staining (P < 0.0001, one-way ANOVA) (Figure 4E, F). We also note a significant increase in the number of apoptotic cells as measured by an increase in the number of TUNEL positive tumor cells (P < 0.001, one-way ANOVA) (Figure 4G, H).

**Combined ATR and Wee1 inhibition is well tolerated**

As the aim of the conditional synthetic lethality is to spare normal tissue, we studied potential toxicities in treated mice, particularly in tissues with fast-proliferating cells and relying on stem cells for regeneration. We first tested tumor bearing immune-compromised NSG mice (n = 9 per group), used for our xenografts, and immune-competent C57BL/6 mice (n = 6 per group) for rudimentary indicators of side effects after treatment with 25 mg/kg AZD6738 and/or 60 mg/kg AZD1775 daily over a period of 26 days (Figure 5A). None of the mice showed significant changes in body weight (Figure 5B, C), behavior (including food intake) or feces consistency (data not shown). Post mortem analyses revealed no signs of inflammation or changes in spleen size. Only one mouse (in the ATR alone treatment group) showed signs of partial hair loss (Supplementary Figure 13).
Due to renewal kinetics, tissues relying on fast-proliferating cells are particularly endangered by drugs that increase replication stress. Crypt intestinal stem cells support the continuous regeneration of the small intestine epithelium, the most rapidly self-renewing tissue in adult mammals (39). We therefore examined intestinal cells for DNA damage and measured the villi length of ilea from NSG mice. Due to abrasion villi are constantly replenished by the progenitor cells sitting in the crypt. Although we see an increase in crypt cells staining for γH2AX (Figure 5D) in mice treated with Wee1 inhibitor alone or in combination, but not with ATR inhibitor alone, no treatment group showed a decrease in villi length by day 26 (Figure 5E). In mice crypt stem cell depletion, e.g. by ionizing radiation, can lead to observable changes in villi within 4 days (40), yet the combination treatment over a period of 26 days is well tolerated in our mouse intestines, in agreement with no signs of diarrhea or changes in body weight. To test for changes in another tissue sensitive to genotoxic stress, we isolated the bone marrow from immune-competent C57BL/6 mice after 26 days of inhibitor treatment. Bone marrow injury is one of the most common dose-limiting adverse effects of cancer therapy with genotoxic agents. Radiation and chemotherapy induce hematopoietic cell apoptosis, particularly in multipotent progenitor and hematopoietic progenitor cells, which proliferate and have lower DNA repair capacity than the quiescent hematopoietic stem cells they derive from (41). Hematopoietic stem and progenitor cells can be identified by surface markers (42, 43). We used flow cytometry to quantify stem and progenitor cells from bone marrow using two different marker combinations, CD117+/Sca1+ (hematopoietic stem and multipotent progenitor cells) (Figure 5F, G) and CD117+/Lin- (which additionally include myeloid progenitor cells) (Figure 5H, I) (41). We did not see any significant changes in the percentage of these subpopulations in bone marrows from inhibitor treated mice compared to control mice (Figure 5G, I).
To more rigorously test for drug-induced damage to normal tissue we harvested additional tissues from tumor bearing immune-compromised NSG mice (n = 3 per group) and immune-competent C57BL/6 mice (n = 3 per group) immediately after the 26 day (“26 d”) drug treatment, or one (“33 d”) or two weeks after the last day of drug administration (Figure 6 and data not shown). Only in the ileum and in the spleen of mice at the end of the drug treatment did we observe an increase of γH2AX-staining cells. No DNA damage was observable e.g. in lungs, kidneys or livers from either NSG or C57BL/6 mice (Figure 6 and Supplementary Figure 11). Remarkably after a period of just 7 days after last drug administration, the number of DNA damaged cells in the ilea or the spleens returned to background levels (Figure 6, left panel). We also analyzed the blood of mice at the end of and 1 or 2 weeks after drug treatment. Pathological evaluations did not reveal any significant changes in complete blood cell count (Supplementary Table 1 and Supplementary Table 2), in agreement with the lack of observable hematopoietic stem cell depletion (Figure 5 F-I). In summary, the increased endogenous DNA damage in cancer cells compared to even actively proliferating normal cells seems to provide a significant therapeutic window for the combination treatment.

Combined inhibition of ATR and Wee1 leads to tumor remission, increased survival and inhibition of metastasis

To test drug efficacy in longitudinal studies using our xenograft model, once tumors reached a volume of 40-50 mm³ mice were randomly allocated to treatment or vehicle arms (n = 9 mice per treatment group). These mice were administered 25 mg/kg AZD6738 and/or 60 mg/kg AZD1775 daily over a period of 26 days (Figure 5A, yellow shades in Figure 7B, C, F) and tumor growth was followed by caliper measurement every second day and metastasis by weekly inspection with a bioluminometer (Figure 7A). We observed significant inhibition of tumor growth (P < 0.0001,
two-way ANOVA) (Figure 7B) by treatment with AZD6738 and AZD1775. While treatment with AZD6738 or AZD1775 alone delays tumor growth, tumor expansion resumes rapidly when drug treatment is stopped. However, combination treatment leads to tumor shrinkage below 1 mm³, in 6/9 cases even to complete remission as measured by impalpable tumor levels. Although we have not observed complete eradication so far (the high sensitivity of bioluminescence allows for the visualization of residual MDA-MB-231-fluc2), we speculate that a proportionate level of cell killing in immunocompetent patients could lead to tumor control. In our immunocompromised NSG mice that have been treated with the inhibitor combination, tumors do eventually recur (Supplementary Figure 14). Nevertheless, and although mice were only treated for 26 days, mice treated with AZD6738+AZD1775 lived significantly longer ($P < 0.0001$, Log-rank Mantel-Cox; median survival after start of treatment: AZD6738 – 60 days; AZD1775 – 62 days; AZD6738+AZD1775 – 103 days; vehicle control treated – 53 days) (Figure 7C), paralleling the cancer-selective synthetic lethality observed in vitro.

Tagging MDA-MB-231 cells with firefly luciferase also allowed us to follow metastasis by non-invasive bioluminescence imaging. As can be seen by representative images of mice at week 7 (16 days after the last drug administration) and the statistical analysis of bioluminescence at distant sites, combined Wee1 and ATR inhibition strongly suppressed metastasis (Figure 7D). While Wee1 or ATR inhibitor stand-alone treatment did not show any significant inhibition of metastasis, bioluminescence levels at distant sites in the combination treatment are below the background threshold ($P = 0.0383$, one-way ANOVA) (Figure 7E).

To further investigate inhibition of metastasis we treated a set of mice (n = 4 mice per treatment group) only when tumors reached a volume of around 250 mm³. At that point micrometastasis should already have occurred, as the corresponding time relates to approximately 4-5 weeks later
in tumor growth compared to the previous experiments (Compare tumor volumes and metastasis at week 7 for control mice in Figure 7B and D). Again, mice were randomly allocated to treatment arms, consisting of a 26 day period of daily administration of 25 mg/kg AZD6738 and/or 60 mg/kg AZD1775. As seen before, AZD6738 and AZD1775 single treatment led to tumor growth delay ($P < 0.0001$, two-way ANOVA), but combined treatment led to tumor shrinkage ($P < 0.0001$, two-way ANOVA) (Figure 7F). Secondary tumors were observed in the thoracic lymph nodes of control, AZD6738 or AZD1775 treated mice, but not in animals receiving the combination treatment ($P = 0.0061$, one-way ANOVA) (Figure 7G). Even more compelling, tissues from the mice euthanized at the end of the treatment were inspected for micrometastases by bioluminescence, a technique that allows us to detect clusters of as few as 20 cells. Unlike in the case of control or single inhibitor treated mice, which showed metastasis to lymph nodes, lungs, liver, bone, gut, and in some case also to the brain and ovaries, tissues from combined ATR and Wee1 inhibitor treated animals showed no detectable micrometastases (Table 2).

**ATR and Wee1 activity are critical for breast cancer stem cell survival**

Our observation that combined ATR and Wee1 inhibition suppresses metastasis of highly invasive MDA-MB-231 (Figure 7D-F and Table 2) could be explained by inhibition of the process of metastasis per se or a depletion of cells able to spread and to initiate tumors at distant sites. Breast cancer stem cells have been implicated in metastasis due to their high cellular plasticity, enabling them to undergo epithelial-mesenchymal transition, and their tumor initiating potential. This prompted us to isolate a subpopulation enriched in cancer stem cells from cell lines of two different breast cancer subtypes, MCF7 (luminal B) and MDA-MB-231 (claudin low), by their dye efflux propensity (44) (Figure 8A). Cancer stem cells often show upregulation of transporter proteins in the ATP-binding cassette family, such as ABCG2. Confirming the stem cell character of the
isolated subpopulation, a much lower number of seeded cells from the fraction with high dye efflux capacity (“side population”, SP) is required to form mammospheres than cells with low efflux capacity (“non-side population”, NSP) (Table 3 and Figure 8B). We next compared cooperative cell killing by ATR and Wee1 inhibitors in the cancer stem cell-enriched side population to a non-side population. MCF7 and MDA-MB-231 cancer stem cells (SP) are more resistant than the control subpopulation (NSP) to either AZD6738 or AZD1775 alone, but surprisingly showed similar sensitivities to the combined treatment (Figure 8C, D). This unexpected finding is due to higher synergistic effects in cancer stem cells than in cancer cells without stem cell features (e.g. lower Bliss combination indices (CI) at 300 nM AZD6738 and 100 nM AZD1775 of 0.40 versus 0.90 for MCF7 and 0.41 versus 0.75 for MDA-MB-231). To our knowledge this is the first reported observation of increased synergistic effects of cytotoxic agents in cancer stem cells compared to bulk cancer cells. The increased synergy in cancer stem cells, although they are more resistant to the single agents, could explain the strong anti-metastatic effect by the combination treatment observed in our animal model.
Discussion

Tumor heterogeneity constitutes one of the biggest barriers to effective cancer therapies. Therapies merely targeting the bulk of cancer cells are often destined to fail because induced clonal drifts and the formation of dormant cells decrease tumor control probability. Furthermore, activation of alternative pathways to counteract targeted therapies can lead to drug resistance. Here we describe a strategy designed to take advantage of the cancer-intrinsic property of DNA damage (8), a feature shared by all clones (albeit potentially to different extents). Genomic instability is a driver of tumorigenesis and has been designated as a hallmark of cancer (45). Cancer cells typically show oncogene-driven genome changes such as an aberrant number or structure of chromosomes (chromosomal instability), microsatellite instability, and/or the mutagenic load. While the impairment of checkpoints that should prevent these events are drivers of tumorigenesis, the increase in accumulated DNA damage leads to replication stress (46) and a high risk of mitotic failure, making the survival of cancer cells heavily relying on an often partially defective DNA damage response.

The gene products of Ataxia telangiectasia mutated (ATM) and ATR are apical kinases of pathways activated by DNA damage. Unlike ATM, which is frequently lost in cancers (47), ATR is an essential gene for the response to DNA damage (21, 22) and ATR activity is often upregulated in cancer cells (48, 49). ATR activation is important for DNA damage repair by homologous recombination (10-12). Furthermore ATR (via Chk1) together with Wee1 negatively regulates the activity of CDK2 and especially of CDK1, the only essential CDK in mammals (50). Likely due to the reliance of cancer cells on the G2/M checkpoint to protect them from mitotic catastrophe as a consequence of excessive DNA damage, Wee1 was found upregulated in several cancer types.
The importance that Wee1 and ATR were found to have in cancer cell survival make them attractive therapeutic targets.

A model for the synergistic cell killing by ATR and Wee1 inhibition

Here we report cancer-selective synergistic killing by ATR and Wee1 inhibition. (While this manuscript was in preparation, another group reported synergistic killing of triple-negative breast cancer cells by Wee1 and ATR inhibitors. (52)) Our data support a model, where synergistic killing by ATR and Wee1 inhibitors is triggered by Wee1 inhibition-induced DNA damage during replication, abrogation of ATR-mediated S phase checkpoint activation, inhibition of ATR-dependent homologous recombination, and amplified by increased entry into mitosis with defective genomes due to combined inhibition of ATR and Wee1 (Supplementary Figure 15).

High replication stress in cancer cells could be due to the high level of baseline DNA damage per se, but also to the resulting exhaustion of factors needed for both repair and replication, such as RPA (53). ATR plays an essential role for cancer cells to survive replication stress. Already hypersensitive to ATR inhibition, we propose that Wee1 inhibition leads to even higher replication stress in cancer cells making them unable to avoid DNA damage during replication at ATR inhibitor doses tolerable to the animals (or patient). Highly proliferative normal tissues by contrast do not have such high baseline replication stress and can tolerate the combination treatment (Figure 8E). This model is supported by our observation that reversal of ATR or Wee1 inhibition alone following S phase leads to minimal cell death (Figure 3D), indicating that the resulting increase in replication stress can be rescued by repair before entry into mitosis. Combined inhibition during replication on the other hand, even if reversed after S phase, leads to substantial cell killing, likely due to extensive genome damage that cannot be repaired before cells enter mitosis. Similarly, combined ATR and Wee inhibition after S phase completion leads to extensive
cell death. This might be due to G2/M checkpoint abrogation and the consequent premature entry into mitosis with unrepaired endogenous DNA damage, but also to functions of ATR and Wee1 during mitosis. ATR was reported to contribute to faithful chromosome segregation by promoting Aurora B activation at centromeres (54). Also Wee1 has a role in mitosis beyond regulating the G2/M checkpoint, as residual Wee1 (potentially together with ATR) inhibits CDK1 activity in anaphase, which controls mitotic exit (9). The abrogation of ATR and Wee1 activity during different phases of the cell cycle cooperatively leads to cell death caused by mitotic defects (Figure 8F). Cell death can occur in mitosis or in interphase after aborted or completed mitosis. As a consequence of coordinated effects that Wee1 and ATR have on faithful cell cycle progression, particularly in cells with high baseline DNA damage, a therapeutic window opens to lower the activity of these two kinases to levels lethal for cancer cells, but tolerable to normal tissues. This is in stark contrast to Chk1 inhibition, which - particularly when combined with Wee1 inhibition (Supplementary Figure 2) - shows high toxicity in non-transformed cells. As previously pointed out by us and others, Chk1 inhibition is not phenotypically identical with ATR inhibition (10, 25, 55).

Cancer-selective synthetic lethality, tumor remission and inhibition of metastasis

Our preclinical mouse data indicate that at doses leading to strong tumor shrinkage, combined ATR and Wee1 inhibition shows minimal adverse effects. The absence of diarrhea or villi change in the ilea as well as of a significant loss of hematopoietic stem and progenitor cells, indicators of intestinal damage or bone marrow injury respectively, suggest that tissues relying on fast proliferating cells for homeostasis are less sensitive to the combination treatment than tumor cells. Phase I studies of AZD6738 (as well as other ATR inhibitors) are currently being undertaken. AZD1775 (currently the only Wee1 inhibitor in clinical development) has already progressed to
several phase II trials, usually in combination with genotoxic agents such as carboplatin or gemcitabine (55). The strong synergistic effects on tumor control described here, leading to complete remission in 6/9 cases by the AZD6738/AZD1775 combination treatment, provide an ideal base for phase I clinical trials. Even more striking is our observation that combined Wee1/ATR inhibition leads to a strong inhibition of metastasis. We observe both inhibition of tumor spread by a 26 day AZD6738/AZD1775 treatment started when tumors are still small as well as the absence of metastatic lesions following the same treatment in mice, when the treatment was initiated after micrometastasis already has happened (Figure 7D-G and Table 2). This observation could be explained by our surprising finding that breast cancer stem cell-enriched populations, although more resistant to either ATR or Wee1 inhibition alone, show a higher synergy in cell killing by co-treatment with AZD6738 and AZD1775, than bulk cells. Cancer stem cells from a variety of tissues were found to display elevated radiation and chemoresistance (56). Interestingly, glioma, colon and lung cancer stem cells were found to have a stronger ATR response to genotoxic agents than bulk cancer cells (57-59), and glioma stem cells were found to be more sensitive to Wee1 inhibition than neural stem cells (60). It could be that the reliance of cancer stem cells on ATR or Wee1 to withstand genotoxic insults makes them particularly vulnerable to combined Wee1/ATR inhibition. Because metastasis is the main cause of death in cancer patients, the anti-metastatic activity and the propensity to kill cancer stem cells could make a combined AZD6738/AZD1775 regimen suitable for stand-alone treatment or for adjuvant therapy.

**Potential strategies for patient selection**

Unlike recently reported by Jin et al. (52), we observe in vitro synthetic lethality of ATR and Wee1 inhibition not only in triple-negative breast cancer cells, but in a wide range of breast cancer cell
lines, including luminal A and B cells (MCF7, T47-D, MDA-MB-175-VII, Zr-75-1) and Her2-
amplified Sk-Br-3. While Jin et al. speculate that p53 mutation sensitizes cancer cells to combined
ATR/Wee1 inhibition, we noticed strong synthetic lethality also in p53 wild type cells, such as
MDA-MB-175-VII, Zr-75-1, and MCF7, and the osteosarcoma cell line U-2 OS. Based on our
model, where ATR and Wee1 inhibition leads to decreased S, S/G2 and G2/M checkpoint
activation, supported by the recent finding that ATR is an important regulator of the S/G2
checkpoint (61), and subsequently leads to mitotic catastrophe, we speculate that p53 status is less
of a predictor of therapeutic outcome by the drug combination than baseline levels of DNA damage
and alterations in the mechanisms regulating CDK1/2 activity. Indeed, our unpublished data show
that factors besides Chk1 and Wee1 regulating CDK1 activity, such as the Wee1-related kinase
Myt1, or factors involved in processing replication stress intermediates play important roles in
cellular sensitivity to Wee1/ATR combination treatment in vitro. Initial clinical trials are expected
in cancer types known for their genomic instability, such as cancers with ATM loss, which we
previously showed to sensitize to ATR inhibition (10), and certain breast, ovarian or colorectal
cancers, where Homologous Recombination (e.g. by BRCA loss) or other repair pathways are
impaired. Yet the conditional synthetic lethality underlying combined AZD1775/AZD6738
treatment is based on the increased DNA damage per se in cancer cells compared to normal tissue.
This genotoxic stress can have various origins, from aneuploidy to gene or epigenetic defects, but
will result in replication stress. Unfortunately, while several candidate predictive biomarkers have
been identified for cellular sensitivity to ATR or Wee1 inhibitors (reviewed in (62)), clinical
biomarkers for DNA replication stress are still lacking. Whereas in vitro FANCD2 or RAD51 foci
resulting from the recruitment of these proteins to common fragile sites are good surrogate markers
for replication stress (63, 64), attempts to use Ki-67, cyclin E, POLD3, γH2AX, and FANCD2
staining in cancer specimens by IHC have been disappointing (65). Incidentally ATR activation should correlate with replication stress and future studies will assess whether ATR phosphorylation at T1989 in cancer biopsies, used as marker of ATR activation in our xenografts, is a predictive biomarker for combined ATR/Wee1 inhibitor treatment.
Methods

Cell synchronization and cell cycle analysis: U-2 OS cells were treated with 2 mM thymidine for 16 h, released into fresh medium for 4 h followed by nocodazole (100 ng/mL) treatment for 8 h. Six h after release from nocodazole into fresh medium, cells were treated with DMSO, AZD6738 (1 µM), AZD1775 (0.3 µM), or a combination of AZD6738 and AZD1775.

MDA-MB-231 cells were treated with 2 mM thymidine for 18 h, followed by release into fresh medium for 8 h and a second treatment with 2 mM thymidine for 18 h. After release, cells were treated with either DMSO, AZD6738 (1 µM), AZD1775 (0.3 µM), or combined AZD6738 and AZD1775.

For cell cycle analysis, cells were harvested at 2 h intervals and fixed with 70% chilled ethanol for at least 24 h at -20°C before a wash with 1X PBS. Pelleted cells (1500 rpm, 5 min) were resuspended in propidium iodide (PI) buffer (50 µg/mL) containing RNAse A (10 µg/mL) and incubated at 37°C for 30 min. Samples were analyzed on a BD FACSCanto II flow cytometer.

Orthotopic breast cancer xenografts and drug treatments: All mice were obtained from breeding colonies at the University of Alberta. All animal studies described were carried out under protocol number AC16225 approved by the Cross Cancer Institute’s Animal Care Committee, Edmonton, Canada. For tumor formation, 2 x 10^6 MDA-MB-231-fluc2-tdT cells were mixed with Matrigel (Corning, USA) and PBS (1:1) and injected in 50 µL volume orthotopically into the inguinal mammary fat pad of 6 to 8 weeks old female NOD.Cg-Prkdcsid I2rgtm1Wjl/SzJ (NSG) mice. Tumor growth was measured every 4 days using a Vernier caliper and volume was assessed as [length x (width)^2]/2. When the tumor volumes reached approx. 40-50 mm³, mice were randomly segregated into 4 groups (n = 9 per group). Mice were treated daily with vehicle,
25 mg/kg AZD6738 (in 10% DMSO, 40% polypropylene glycol, and 50% ddH₂O), 60 mg/kg AZD1775 (in 0.5% methylcellulose), or a combination of AZD6738 and AZD1775 via oral gavage for 26 days. Body weight was measured every 4 days as an indicator of toxicity. Mice were euthanized when the tumor volume reached a total of 1000 mm³, after a > 10% reduction in body weight, or any other indications of physical discomfort.

For histological studies, tumor bearing mice (approx. 250 mm³ tumor volume) were treated with either vehicle or 25 mg/kg AZD6738 or 60 mg/kg AZD1775 or a combination of AZD6738 and AZD1775 via oral gavage for 5 (short term) or 26 (long term) days. Tumors and small intestines (ilea), lungs, livers, kidneys, and spleen were harvested and fixed with 10% formalin for 48 h prior to embedding.

**Statistical analysis:** All statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, California, USA). All experiments were performed at least 3 times in triplicates or quadruplets. P-values were calculated using one-way ANOVA, two-way ANOVA, and Log-rank (Mantel-Cox) test. P-values of < 0.05 were considered significant, and P-values of < 0.001 were considered highly significant.

**Study approval:** All animal studies described were carried out under protocol AC16225 approved by the Cross Cancer Institute’s Animal Care Committee, Edmonton, Canada.

Details of Materials and other Methods, such as survival assays, side population and mammosphere assays, immunofluorescence and immunohistochemistry, microscopy, bioluminescence imaging, and hematopoietic progenitor studies are described in Supplementary Information.
Author contributions

ABB, CWL, GKC, and AMG designed the experiments. ABB, CWL, JP, and DL performed crystal violet assays. ABB, CWL, and AMG performed in vitro experiments and analyzed the data. ABB performed in vivo experiments and analyzed the data. ABB performed statistical analysis. GKC and AMG conceived the data. ABB and AMG wrote the manuscript.

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References


Figure 1. Wee1 inhibition activates ATR and shows synergistic cancer cell killing with ATR inhibition. (A) MDA-MB-231 cells where incubated with the indicated inhibitors for Wee1 (AZD1775) or ATR (AZD6738, ETP46464). After 2 hours cell lysates were harvested and probed for Chk1 and CDK1 phosphorylation by immunoblotting. (B, C, D, E, F) MDA-MB-231, MCF7, Zr-75-1, hTERT-HME1, or MCF10A cells were treated for 4 days with a combination of up to 4 μM AZD6738 and up to 2 μM AZD1775. Survival was assayed by Crystal Violet staining and each experiment was repeated at least 4 times. Color bars indicate % survival normalized to untreated cells. Representative cooperativity screens and Loewe plots for drug cooperativity are shown.
Figure 2. Combined ATR and Wee1 inhibition leads to mitotic defects and cancer cell death. (A-D) Live cell imaging of MDA-MB-231 expressing mCherry-histone H2B and GFP-tubulin. (A) Cells treated as indicated (ATRi = 1 μM AZD6738, Wee1i = 0.3 μM AZD1775) were monitored by spinning-disk confocal microscopy. Representative images of cells following nuclear envelope breakdown (NEBD) are shown. (B) Quantification of the time from NEBD to anaphase. (C) Representative fates of 5 cells in the 4 treatment groups. (D) Quantification of observed cell fates (n = 56). Of note, when cell death occurred in interphase, the dying cells had
previously undergone mitosis following drug addition. (E) Representative images of MDA-MB-231 or T-47D mitotic cells treated like in (A). Fixed cells were stained for centromeres (red) and tubulin (green) by immunofluorescence and for DNA with DAPI (blue). Drug-induced clustering of centromeres (white arrows) spatially separated from the main mass of chromosome (yellow arrow), a feature of centromere fragmentation, is clearly visible. (F) Quantification of cells that are in mitosis (red and blue) and display centromere fragmentation (blue) (n > 1000), after fixing cells 4 h after release from a double thymidine block in the presence of the indicated inhibitors. * indicates $P < 0.05$, **** indicates $P < 0.0001$ (one-way ANOVA).
Figure 3. Contribution of cell cycle phases, during which ATR and/or Wee1 was inhibited, to overall cell killing. (A, B) AZD6738 and AZD1775 are reversible inhibitors. Immunoblots of MDA-MB-231 and U-2 OS cells treated as indicated. (A) The ATR inhibitor AZD6738 (1 µM) was added to cells 15 minutes before irradiation with 40 J/m² UV, a strong activator of ATR. 1 h after irradiation AZD6738 was removed, the cells were washed and harvested at indicated times after drug removal. Restoration of ATR activity is observed 1 h after AZD6738 washout. (B) Cells were incubated for 2 h with 300 nM Wee1 inhibitor AZD1775 leading to a strong reduction in phospho-CDK1. AZD1775 was then removed and cells washed, leading to restoration of Wee1 activity within 1-2 h. (C) U-2 OS cells were synchronized by thymidine-nocodazole blocks. 6 h after release cells were treated with 1 µM AZD6738 and/or 300 nM AZD1775. Cell cycle profiles were analyzed by propidium iodide staining and flow cytometry. (D) ATR and/or Wee1 in synchronized cancer cells were transiently inhibited with 1 µM AZD6738 and/or 300 nM AZD1775 during the indicated cell cycle intervals. Survival of drug-treated cells relative to vehicle control was measured after 4 days. Data represents mean ± SD. * indicates \( P < 0.05 \), ** indicates \( P < 0.005 \), and **** indicates \( P < 0.0001 \) (one-way ANOVA).
Figure 4. AZD6738 and AZD1775 inhibit ATR and Wee1 respectively in vivo. MDA-MB-231-fluc2-tdTomato xenografts were excised for immunohistochemistry 1 hour after last administration of the inhibitors to the mice by oral gavage for 5 days (25 mg/kg AZD6738 and/or 60 mg/kg AZD1775 daily). ATR (A) and Wee1 activity (B) was tested by probing for phosphorylation of their respective substrates, ATR Thr1989 and CDK1 Tyr15 (Insets show tumor tissue at 40x magnification). (C, D) DNA damage was tested for with antibodies against γH2AX. Data represents mean ± SD. (E, F) Ki67 staining was used as a readout for proliferating cells. Scale = 100 μm; Inset scale = 25 μm. Data represents mean ± SD. (G, H) TUNEL assay was used to quantify cell death in excised tumor sections. Scale = 20 μm. Data represents mean ± SD. * indicates P < 0.05, *** indicates P < 0.001, and **** indicates P < 0.0001 (one-way ANOVA). (DAB = 3,3'-diaminobenzidine)
**Figure 5. Combination treatment with ATR and Wee1 inhibitors and normal tissue toxicity.**

(A) Mice were treated for 26 days daily with 25 mg/kg AZD6738 and/or 60 mg/kg AZD1775 and tested for adverse effects. (B, C) No significant body weight changes are observed in tumor bearing immune-deficient NSG or in immune-competent C57BL/6 mice. (D, E) Although Wee1 inhibition leads to some γH2AX staining in the crypt of NSG mouse ilea (see insets) (Scale = 100 μm; Inset scale = 25 μm) (D), no significant change in villi length is observed (E). n = 50 refers to 50 measurements in each of 3 mice per group. (F-I) No significant depletion of hematopoietic stem and progenitor cells isolated from treated C57BL/6 mice is observed. Bone marrow cells were isolated from C57BL/6 mice treated as described in (A) and analyzed with the indicated surface markers by flow cytometry. (F, G) Hematopoietic stem and multipotent progenitor cells stain for
CD117+/Sca1+. Data represents mean ± SD. (H, I) The CD117+/Lin- population additionally includes myeloid progenitor cells. Data represents mean ± SD.
Figure 6. Evaluation of normal tissue DNA damage. Tissues from tumor-bearing NSG mice (or immune-competent C57BL/6 mice without tumors, shown in Supplementary Figure 11) were harvest on the last day (“26 d”) or one week after (“33 d”) the last day of a 26 day treatment period with AZD6738 and/or AZD1775. While lung, liver and kidney did not show any signs of DNA damage, some cells in the ileum and spleen were found to stain for γH2AX at the end of the treatment (“26 d”). Yet one week later (“33 d”), also ilea and spleens recovered from the drug treatment, as measured by staining for γH2AX. Scale = 25μm; Inset scale = 20 μm.
Figure 7. Combination treatment with ATR and Wee1 inhibitors and tumor control. (A-E)
NSG mice were injected orthotopically with MDA-MB-231-fluc2-tdTomato labelled breast cancer cells and treated for 26 days (indicated by yellow shades) with 25 mg/kg AZD6738 and/or 60 mg/kg AZD1775 after tumors reached around 40 mm<sup>3</sup>. (A) Tumor progression was monitored weekly by bioluminescence imaging. (B) Tumor growth of mice in the 4 treatment arms (n = 9 per group). Data represents mean ± SD. (C) Kaplan-Meyer survival curves of treated mice (n = 9 per group). (D, E) Metastasis in regions distal to the primary tumor was assessed 7 weeks after treatment initiation (n = 9 per group). The dotted line indicates background threshold (E). (F-G) To further investigate inhibition of metastasis, a group of MDA-MB-231-fluc2-tdTomato tumors (n = 4 per group) were allowed to grow to around 250 mm<sup>3</sup> before treatment like in (A). Combination treatment leads to tumor shrinkage (F). Data represents mean ± SD. Unlike control or single agent treated mice, mice treated with AZD6738 and AZD1775 had no detectable secondary tumors (G). * indicates P < 0.05, ** indicates P < 0.01, **** indicates P < 0.0001 (B, F: two-way ANOVA; C: log-rank Mantel-Cox test; and E, G: one-way ANOVA).
Figure 8. Synergistic killing of breast cancer stem cells by ATR and Wee1 inhibitors. (A) Isolation of cancer stem cell-enriched subpopulations (“side population”, SP) from MDA-MB-231 or MCF7 based on their increased dye (DCV) efflux properties. Verapamil, an inhibitor of drug efflux pump proteins, particularly of the ABC transporter family, served as negative control. (B) Isolated “side populations” show an increased ability to form mammospheres compared to control subpopulations (“non-side population”, NSP). Representative images of mammospheres are shown. (C, D) Four-day survival assays of cancer stem cell enriched “side populations” (SP) and control cells (NSP) isolated from MDA-MB-231 (C) and MCF7 (D). Plated cells were treated with indicated concentrations of AZD1775 and/or AZD6738. Color bars indicate % survival normalized to untreated cells. (E) Model of cancer-selective synergistic cell killing by combined ATR and Wee1 inhibition. Cancer cells have higher baseline levels of genotoxic stress than normal cells. Wee1 inhibition increases genotoxic stress, while ATR and Wee1 inhibition together lower cellular DNA damage response capacity (in the simplified model to the same extent, but potentially higher in cancer cells relying on these two kinases for survival). A therapeutic window is created for the selective killing of cancer cells. (F) Cell cycle-dependent effects of ATR and Wee1 inhibition contributing to overall cell death following mitotic catastrophe.
### Tables

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<th>AZD1775 (Wee1i)</th>
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**Table 1.** IC<sub>50</sub> values and Bliss combination indices (CI) at indicated drug concentrations calculated from at least three independent experiments. A Bliss CI of less than 1 indicates synergy, a CI of < 0.7 strong synergy, and a CI of > 1 antagonism.

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<th>AZD6738 (ATRi)</th>
<th>AZD1775 (Wee1i)</th>
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**Table 2.** Ex vivo bioluminescence imaging of excised tissues revealed micrometastases in several organs from control or single inhibitor treated mice, but no micrometastases were observed in the AZD6738/AZD1775 combination treatment group.
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Table 3. Isolated “side population” cells demonstrate higher mammosphere forming capabilities as compared to the “non-side population” cells.