# Chemotherapy-induced differential cell cycle arrest in B-cell lymphomas affects their sensitivity to Wee1 inhibition

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**ABSTRACT**

Chemotherapeutic agents, e.g., cytarabine and doxorubicin, cause DNA damage. However, it remains unknown whether such agents differentially regulate cell cycle arrest in distinct types of B-cell lymphomas, and whether this phenotype can be exploited for developing new therapies. We treated various types of B cells, including primary and B lymphoma cells, with cytarabine or doxorubicin, and determined DNA damage responses, cell cycle regulation and sensitivity to a Wee1 inhibitor. We found that cyclin A2/B1 upregulation appears to be an intrinsic programmed response to DNA damage; however, different types of B cells arrest in distinct phases of the cell cycle. The Wee1 inhibitor significantly enhanced the apoptosis of G2 phase-arrested B-cell lymphomas by inducing premature entry into mitosis and mitotic catastrophe, whereas it did not affect G1/S-phase-arrested lymphomas. Cytarabine-induced G1-arrest can be converted to G2-arrest by doxorubicin treatment in certain B-cell lymphomas, which correlates with newly acquired sensitivity to the Wee1 inhibitor. Consequently, the Wee1 inhibitor together with cytarabine or doxorubicin inhibited tumor growth *in vitro* and *in vivo* more effectively, providing a potential new therapy for treating B-cell lymphomas. We propose that the differential cell cycle arrest can be exploited to enhance the chemosensitivity of B-cell lymphomas.

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## Introduction

Cytarabine, known as Ara-C, rapidly converts to cytosine arabinoside triphosphate, which can be incorporated into DNA during the process of DNA synthesis, and eventually causes DNA damage, probably by stalling replication forks and generating DNA double-stranded breaks. Given that cancer cells proliferate rapidly, Ara-C can kill cancer cells by interfering with their DNA synthesis during the S phase of the cell cycle. Ara-C has been the backbone of induction chemotherapy for acute myeloid leukemia and acute lymphocytic leukemia for several decades.[1,2] For non-Hodgkin lymphomas, Ara-C is used as an upfront therapy for mantle cell lymphoma and Burkitt lymphoma, and as part of some salvage regimens when non-Hodgkin lymphomas relapse. However, it remains incompletely understood how Ara-C treatment regulates DNA damage responses in primary B cells and B-cell lymphomas.

The current treatment of B-cell non-Hodgkin lymphomas typically includes R-CHOP, a combination of anti-CD20 (rituximab), three chemotherapy agents (cyclophosphamide, doxorubicin, vincristine), and one steroid (prednisone).[3,4] This regimen has increased the rates of complete response for both young and elderly patients with diffuse large B-cell lymphoma.[5,6] Both cyclophosphamide and doxorubicin are also DNA-damaging agents, although their functional mechanisms are different from those of Ara-C. Doxorubicin is commonly used to treat cancers, including breast cancer, bladder cancer, lymphoma and acute lymphoblastic leukemia.[7]

Doxorubicin can stabilize the complex of topoisomerase II and broken DNA strands, thereby preventing the broken DNA double helix from being resealed and causing stalled DNA replication. Furthermore, the formation of doxorubicin-DNA adducts could activate DNA damage responses independent of topoisomerase II.[8] When cells experience DNA damage, the cell cycle can be arrested in the G1, S or G2 phase for DNA repair.[9] If the DNA damage is beyond recovery or the level of double-stranded breaks exceeds the repair capacity, cells never enter mitosis but die or undergo senescence.[9] It does, however, remain poorly understood how doxorubicin treatment regulates cell cycle arrest and cell death in B-cell lymphomas.

Cell cycle checkpoints are critical to control the progression of the cell cycle of DNA-damaged cells. The active complex of CDK1 and cyclinB1 controls entry into the mitotic (M) phase, and the expression of CDK1 is constitutive. Tyr15 phosphorylation mediated by Wee1 and Myt1 would inactivate CDK1, thus inhibiting mitotic entry. CyclinB1 expression increases at late S phase and reaches the peak at late G2 phase. CyclinB1 down-regulation would arrest cells at G2 phase, thus reducing mitotic entry.[10,11] Further study proved that cyclinB1 is rate limiting but not essential for mitotic entry and progression.[12] Abrogation of the G2/M checkpoint, for instance, by reducing the phosphorylation level of CDK1, enhances premature mitotic entry upon DNA damage, leading to increased cell death via mitotic catastrophe.[9,13] Previous studies have shown that combined treatment with genotoxic drugs and Wee1 inhibitor efficiently controls leukemia progression.[14-16] It remains unclear whether Wee1 inhibitor enhances the M phase entry of cell cycle-arrested B-cell lymphomas and, if so, whether G1, S or G2 phase-arrested lymphomas are sensitive to Wee1 inhibitor.

In the current study, we employed primary mouse B cells, and various mouse and human B-cell lymphoma lines to test how B cells respond to Ara-C or doxorubicin treatment and to elucidate the relationships among DNA damage, cell cycle arrest and the cell death pathway. Our data suggest that cyclinB1/A2 upregulation is an intrinsically programmed DNA damage response. We show that different types of B cells exhibit differential cell cycle arrest upon Ara-C or doxorubicin treatment. Overall, our studies may reveal new mechanistic insights into DNA damage responses and cell cycle regulation, identify biomarkers to predict chemosensitivity and facilitate the development of novel therapies for B-cell lymphomas and beyond.

## Methods

### Cell culture and SOMAscan assay

CH12 lymphoma cells were cultured as described previously.[17] G1XP lymphomas were generated, established and cultured as described previously.[18] Ramos, OCI-LY1, OCI-LY3, OCI-LY7 and DHL-16 were gifts from Dr. Wing C. Chan (University of Nebraska, NE, USA) and were cultured in 10% fetal bovine serum lymphocyte medium. Lymphoma cells were cultured at $0.5\times10^6$/mL, and treated with Ara-C (Cat. SY004943, Accela, San Diego, CA, USA), MK1775 (Cat. 2373, Biovision, Milpitas, CA, USA) or doxorubicin (Cat. 159101, MP Biomedicals) at indicated concentrations for 6 h or 24 h. Splenic B cells were isolated from wildtype (wt) naïve mice using a negative selection kit (Stem Cell Technologies, Canada), cultured with anti-CD40 and interleukin-4 as described previously,[19] and collected 4 days after culture for Ara-C treatment or for western blot and flow cytometry analysis. Details of the SOMAscan assay and computational analysis are provided in the *Online Supplementary Materials and Methods*.

### Western blot, flow cytometry, and knockdown of cyclins

Primary antibodies used in the western blots are listed in *Online Supplementary Table S3*. Secondary horseradish peroxidase-conjugated anti-mouse, anti-goat and anti-rabbit antibodies were from Jackson ImmunoResearch (West Grove, PA, USA) and developed by ECL™ western blotting detection reagents (GE Healthcare, Little Chalfont, UK) according to the instructions provided with the kit. Details of the flow cytometry analysis including cell cycle analysis and cyclin knockdown are given in the *Online Supplementary Materials and Methods*.

### Fluorescence microscopy

Cells were collected and placed on poly-L-lysine-treated cover slips for 30 min, fixed by 4% paraformaldehyde for 1 h at room temperature and permeabilized by 0.1% Triton X-100 for 30 min. After blocking with 2% bovine serum albumin for 30 min, cells were covered by Vecta shield mounting medium (Cat.H-1200, Vector Laboratories, Burlingame, CA, USA) and slides. Images were acquired with an Eclipse TE2000 (Nikon).

### *In vivo* treatment of the transplant G1XP lymphoma model

G1XP lymphomas were generated by crossing Cγ1Cre knock-in, *Xrcc4*, and *Trp53* conditional knockout mice, as described previously.[18] Animal work was approved by the Institutional Animal Care and Use Committee of University of Colorado Anschutz Medical Campus (Aurora, CO, USA). Details of the treatment are provided in the *Online Supplementary Materials and Methods*.

## Results

### Ara-C treatment induced apoptosis via caspase3 activation and DNA fragmentation

Ara-C is analogous to deoxycytidine and is thought to be an S phase-specific agent.[20,21] How Ara-C causes the death of B cells remains incompletely understood. We treated mouse B-cell lymphoma, CH12 cells,[22] with Ara-C, and found that Ara-C increased the percentage of annexin-in-V-positive CH12 cells and caused DNA fragmentation (*Online Supplementary Figure S1A-C*). Since the occurrence of DNA fragmentation is related to caspase3-induced apoptosis, we examined whether Ara-C treatment affected caspase3 activation. Our data showed that cleaved caspase3 was increased in a dose-dependent manner upon Ara-C treatment (*Online Supplementary Figure S1D,E*). Recent studies found that chemotherapeutic agents can cause necroptosis, a regulated form of necrosis or inflammatory cell death.[23] We, therefore, examined the expression of RIP3 (RIPK3) and CaMKII, essential components of the necroptosis pathway, upon Ara-C treatment.[24-26] We found that Ara-C did not increase RIP3 or CaMKII in various B-cell lymphomas (*Online Supplementary Figure S1F*). We, therefore, conclude that Ara-C causes apoptosis by activating caspase3 in B-cell lymphomas and does not induce necroptosis.

### Ara-C treatment upregulated cyclinB1 and cyclinA2 in various types of B cells

To elucidate how primary B cells or B-cell lymphomas respond to DNA damage, we employed cutting-edge

aptamer-based multiplexed proteomic technology,[27] SOMAscan, to identify the differentially expressed proteins in untreated *versus* Ara-C-treated B cells. Due to the breakthrough of SOMAmer,[27] the SOMAscan™ proteomic assay enables us to quantify 1310 proteins across approximately eight logs of concentration as shown by previous studies.[28,29] Among 1310 proteins tested, we found that there were only three proteins differentially expressed between untreated and Ara-C-treated primary B cells from wt or *Xrcc4/p53* double conditional knockout mice.[18] These were cyclinB1/cdk1, cyclinA2/cdk2 and importin B1 (IMB1), all of which were significantly upregulated (fold change ≥2, $P<0.05$) in Ara-C-treated primary activated B cells (Figure 1A and *Online Supplementary Tables S1 and S2*). We present the top ten upregulated/downregulated proteins in Ara-C-treated wt or double conditional knock-out primary B cells (*Online Supplementary Figure S2A,B*).

We recently established a unique mouse model by specifically deleting a NHEJ gene, *Xrcc4*, and *Trp53*, in ger-minal center B cells, which results in the spontaneous development of G1XP lymphomas.[18] Cell lines were established from G1XP lymphomas. In line with our data from primary B cells, we found that cyclinB1 and cyclinA2 were also upregulated by Ara-C in our newly developed G1XP lymphomas and in CH12 lymphomas (Figure 1A).

We performed western blotting to validate the results of the SOMAscan assay. Consistently, we found that cyclinB1 and cyclinA2 were indeed upregulated in Ara-C-treated wt or p53 conditional knock-out primary B cells, and in CH12 or G1XP lymphoma cells (Figure 1B). Notably, we found that CDK1 phosphorylation (pCDK1) at Tyr15 was also enhanced by Ara-C treatment in primary B cells or B lymphoma cells; in contrast, total protein expression of CDK1 and CDK2 was not increased (Figure 1B). When CDK1 is phosphorylated at Tyr15 by Wee1 kinase, it is inactivated and blocks M phase entry. Thus, these data suggest that although cyclinB1 is upregulated, the cyclinB1/CDK1 complex stays inactive and
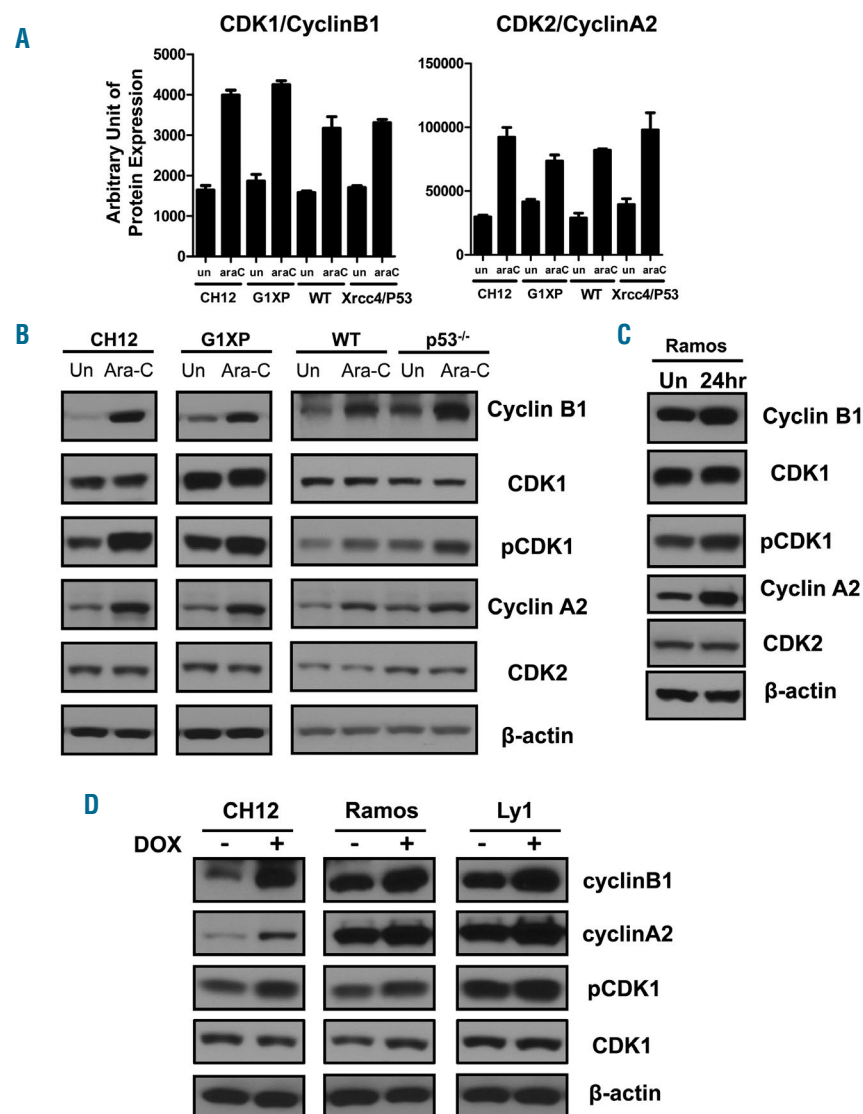


Figure 1. Upregulation of cyclinB1 and cyclinA2 in different types of B cells upon Ara-C treatment. (A) SOMAscan analysis of untreated or Ara-C-treated B cells. CH12 lymphoma (n=3), G1XP lymphoma (n=3), wt (n=3) or *Xrcc4/p53* conditional knockout (n=3) primary B cells were treated with 10 μM Ara-C for 16 h. Cell lysates were prepared and subject to SOMAscan assay. Data are presented as mean±s.e.m. (B) Increased expression of cyclinB1, cyclinA2 and pCDK1 in different types of B cells upon Ara-C treatment. CH12 or G1XP lymphomas, wt or p53^{-/-} primary B cells were treated with 1 μM Ara-C for 24 h. CyclinB1, CDK1, pCDK1, cyclinA2 and CDK2 were detected by western blot and β-actin as the loading control. (C) Ramos lymphomas were treated and analyzed as described in (B). (D) Upregulation of cyclinB1, cyclinA2 and pCDK1 in different types of B-cell lymphomas upon doxorubicin (DOX) treatment. CH12, Ramos and Ly1 lymphomas were treated with 100 nM doxorubicin for 24 h and analyzed as described in (B). Data are representative results of three independent experiments.

probably would not promote M phase entry (see below).

To generalize our findings, we treated several human B-cell lymphoma lines, including Ramos, OCI-LY1, OCI-LY3, OCI-LY7 and DHL-16, and found that Ara-C treatment upregulated cyclinB1, cyclinA2, and pCDK1 expression in all of them (Figure 1C, *Online Supplementary Figure S3A*). Additionally, we examined other players involved in DNA damage responses and found that Ara-C treatment activated Chk1 and Chk2 in most of the lymphoma lines examined. However, we did not detect obvious differences in the levels of CDC25A or pCDC25C (*Online Supplementary Figure S3B*). Next, we examined the kinetics of cyclinB1/A2 upregulation. CyclinB1 and cyclinA2 were upregulated after 8 h of Ara-C treatment (*data not shown*), and significant induction occurred after 20 h or 24 h (*Online Supplementary Figure S3C*). Overall, our results showed a time-dependent effect of Ara-C treatment on upregulating cyclinB1/A2.

Lastly, we showed that another DNA-damaging agent, doxorubicin, can also upregulate cyclinB1/A2 in several B-cell lymphoma lines (Figure 1D). We conclude that cyclinB1 and cyclinA2 upregulation appears to be an intrinsically programmed DNA damage response, which occurs in both activated primary B cells and various B-cell lymphomas.

## Ara-C induces differential cell cycle arrest in different types of B cells

Since cyclins control cell cycle progression, we examined how Ara-C affected cell cycling in mouse primary B cells and B-cell lymphomas in a time- and dose-dependent manner. Based on the Ara-C doses employed in previous studies,[30] we chose to test the effects of treatment with 1 µM and 10 µM Ara-C. Six hours of Ara-C treatment did not affect cell cycle progression significantly in wt primary B cells regardless of Ara-C dosage (Figure 2A, top panel). In contrast, after 24 h of treatment, wt primary B cells predominantly arrested in the S phase in the presence of 10 µM Ara-C, whereas, 1 µM Ara-C treatment caused a modest increase in the percentage of S and G2 phase-arrested
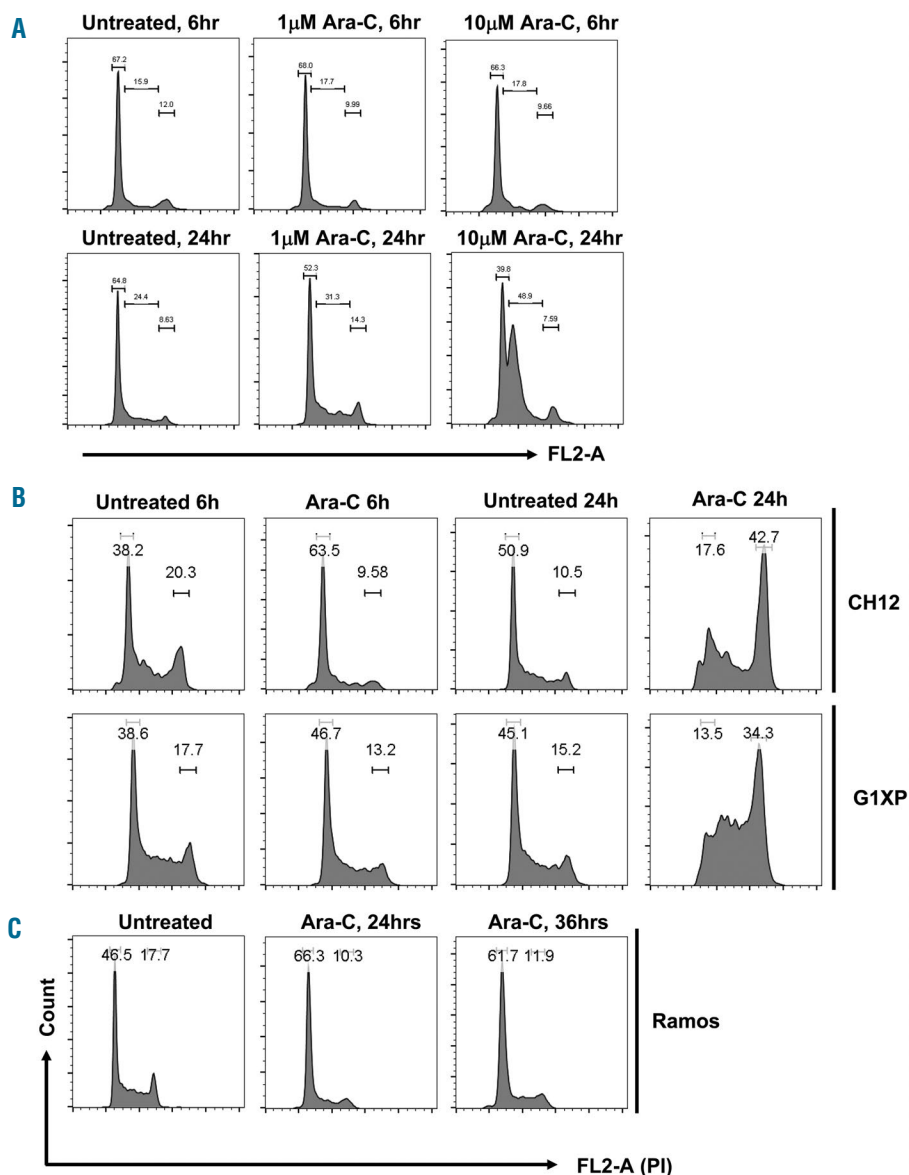


Figure 2. Different types of B cells are arrested in distinct phases of the cell cycle upon Ara-C treatment. (A) Primary B cells were arrested in the S phase of the cell cycle upon Ara-C treatment. Anti-CD40/IL-4 activated wt primary B cells were treated with 1 µM or 10 µM Ara-C for 6 or 24 h. (B) Mouse B-cell lymphomas were arrested in the G2 phase of the cell cycle upon Ara-C treatment. CH12 or G1XP lymphoma cells were treated with 1 µM Ara-C for 6 or 24 h. (C) Ramos lymphoma cells were treated with 1 µM Ara-C for 24 or 36 h. Cells were collected at indicated time points and fixed by 70% ethanol. After propidium iodide (PI) staining, cell cycles were determined by FACS (FL2-A). Data are representative results of three to five independent experiments.

cells (Figure 2A, bottom panel). Taken together, our data show that Ara-C induces mouse primary B cells preferentially arrested in the S phase of the cell cycle.

Contrary to our findings in primary B cells, in CH12 cells we found that Ara-C treatment caused arrest in the G1 phase at an early time point (6 h: untreated 38.2% *versus* Ara-C 63.5%) and in the G2/M phase at a later time point (24 h: untreated 10.5% *versus* Ara-C 42.7%) (Figure 2B, top panel). To further corroborate our findings, we determined the cell cycle progression of G1XP lymphomas upon Ara-C treatment. Consistently, our data showed that Ara-C also arrested G1XP lymphomas in the G1 phase at an early time point and in the G2/M phase at the later time point (Figure 2B, bottom panel). Ara-C treatment resulted in apoptosis regardless of G1 or G2 phase of the cell cycle (*Online Supplementary Figure S4A*). All cells of the sub-G1 phase were positive for cleaved caspase3, indicating their apoptotic phenotypes (*Online Supplementary Figure S4B*). Nevertheless, the ratio among G1/S/G2 remained the same with or without sub-G1 phase included. Since we were more interested in living cells, sub-G1 phase cells were not included in the cell cycle analysis.

To distinguish whether Ara-C induces G2 or M phase arrest, we determined the phosphorylation level of histone3 (pH3), which serves as a marker for M phase entry since chromosome condensation requires H3 phosphorylation. We found that pH3 level was reduced upon Ara-C treatment, demonstrating that Ara-C-treated lymphomas were arrested in the G2 phase but failed to progress into the M

phase (*Online Supplementary Figure S5*). These observations are consistent with our findings that the level of pCDK1 (Tyr15) was increased upon Ara-C treatment (Figure 1B), which would inactivate CDK1 and block M phase entry.

In sharp contrast to our findings in CH12 and G1XP, we found that Ramos cells, a human Burkitt lymphoma line, were preferentially arrested in the G1 phase of the cell cycle (Figure 2C). Thus, Ara-C induces differential cell cycle arrest in different types of B cells, namely, S phase arrest in primary mouse B cells, G2 phase arrest in CH12 and G1XP lymphoma cells and G1 arrest in Ramos cells.

### p53 deficiency does not affect Ara-C-induced cell cycle arrest or M phase blockage

The *TP53* gene is the most frequently mutated gene in human cancers.[31] p53 is capable of inducing cell cycle arrest, apoptosis, or senescence, modulating DNA repair or metabolism, and serving as the guardian of the genome.[32-34] Thus, we tested whether p53 deficiency might affect cell cycle progression upon Ara-C treatment. We treated wt or p53 conditional knock-out primary B cells with Ara-C (1 μM or 10 μM). Our data showed that primary B cells were arrested in the S phase after 24 h of Ara-C treatment regardless of p53 genotype (Figure 3A). Next, we sought to determine whether p53 deficiency affects M phase entry. The percentage of the pH3-positive population is relatively low in primary B cells given that they do not proliferate as fast as lymphomas (Figure 3B, Untreated). In order to increase the percentage of M phase cells, we used col-
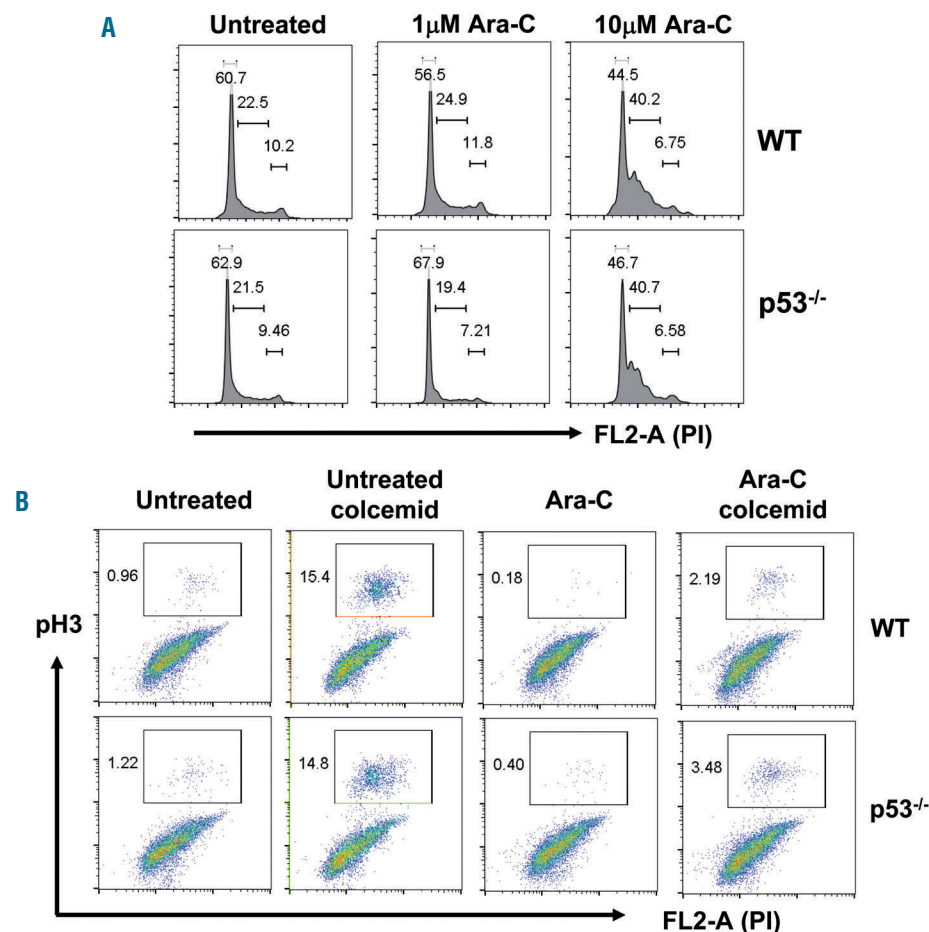


Figure 3. p53 deficiency does not affect the cell cycle changes induced by Ara-C treatment in primary B cells. Wt and p53 conditional knockout primary B cells were treated with 1 μM or 10 μM Ara-C for 24 h. (A) Cell cycles were determined by propidium iodide (PI) staining and (B) mitotic entry was determined by pH3 and PI staining. Data are representative results of three independent experiments.

cemid, a microtubule-depolymerizing drug that blocks M phase progression (Figure 3B, Untreated *versus* Untreated plus colcemid). However, we did not detect a significant difference in cell cycle arrest between wt and p53 conditional knock-out primary B cells (Figure 3B). Ara-C treatment blocked M phase entry regardless of p53 genotype (Figure 3B). We, therefore, conclude that p53 deficiency does not affect cell cycle progression of primary B cells upon Ara-C treatment. This observation is consistent with our findings with G1XP lymphomas that are deficient in both *Xrcc4* and *Trp53*, yet, we found that G1XP lymphomas were arrested in the G2 phase, similar to p53-proficient CH12 cells. These data suggest that Ara-C-induced G2 phase arrest is also independent of p53.

### Increased cyclinA2 and cyclinB1 were not required for Ara-C-induced G2 phase arrest

To address whether increased cyclinA2 and cyclinB1 were required for Ara-C-induced G2 phase arrest,

cyclinA2, cyclinB1 or both were knocked down in CH12 cells by transient transfection of corresponding short hairpin RNA. Western blot confirmed the reduced expression of cyclinA2, cyclinB1, or both in knocked down CH12 cells compared with scrambled controls (*Online Supplementary Figure S6A*). However, the reduction of cyclinA2, cyclinB1, or both had no obvious effects on Ara-C-induced G2 phase arrest (*Online Supplementary Figure S6B*). Additionally, the cleaved caspase3 was not affected by reduced expression of cyclinA2, cyclinB1 or both (*Online Supplementary Figure S6A*).

### Wee1 inhibitor sensitized G2 phase-arrested lymphomas to Ara-C treatment

Ara-C treatment increased pCDK1 (Figure 1B), indicating that pCDK1 may contribute to Ara-C-induced inhibitory effects on mitotic entry (*Online Supplementary Figure S5*). Since Wee1 kinase can phosphorylate CDK1, we employed Wee1 inhibitor to reduce pCDK1 in G1XP
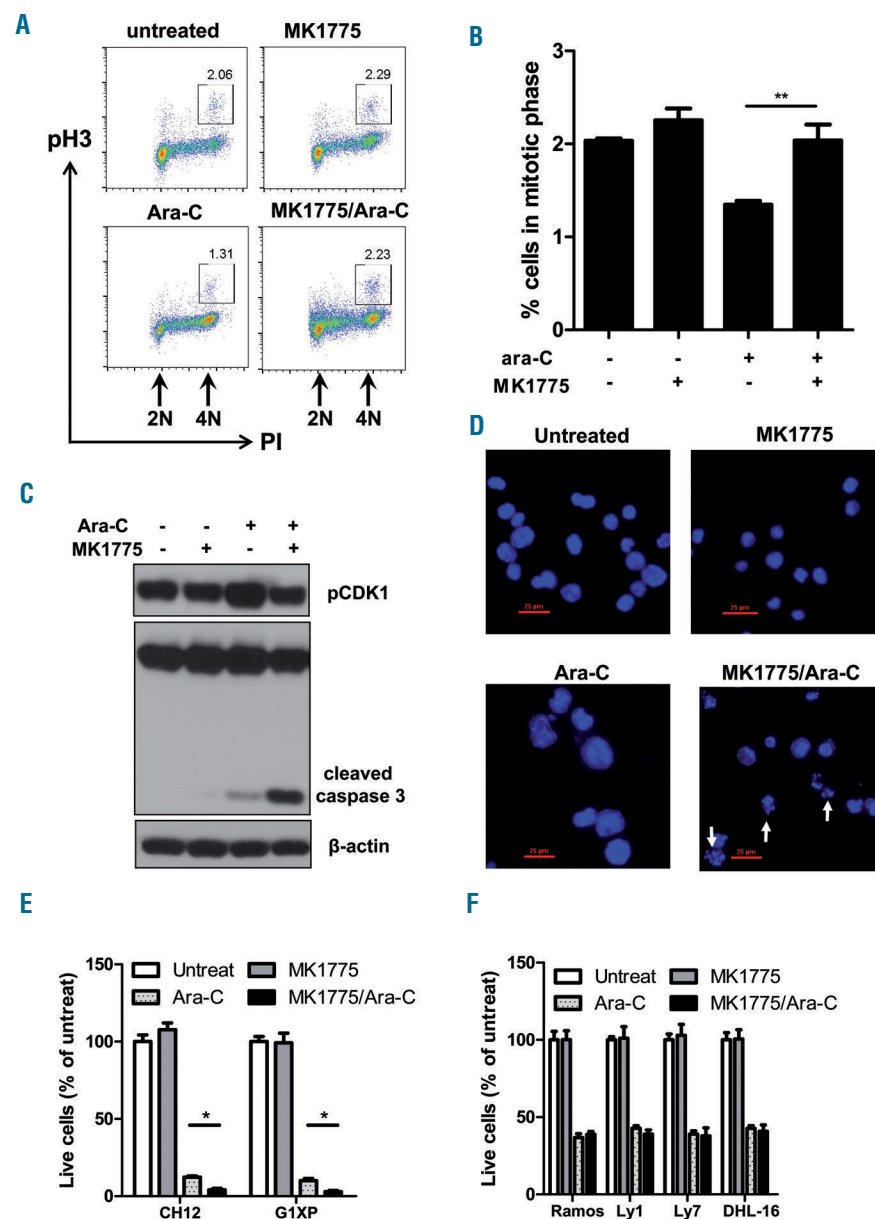


Figure 4. Wee1 inhibitor (MK1775) enhances premature mitotic entry, mitotic catastrophe and apoptosis upon Ara-C treatment. (A, B) Wee1 inhibitor enhances premature mitotic entry of G2 phase-arrested B-cell lymphomas upon Ara-C treatment. G1XP lymphoma cells were either untreated or treated with 1 µM Ara-C, 100 nM MK1775, or both for 24 h. Cells were collected and fixed by 70% ethanol. After propidium iodide (PI) and pH3 staining, cell cycles were determined by FACS (FL1-H/FL2-A). Statistical significance was calculated with one-way ANOVA, Tukey multiple comparison test, **$P \leq 0.01$ in (B). (C) Increased apoptosis of B-cell lymphomas upon combined treatment with Ara-C and MK1775. G1XP lymphoma cells were treated as described in (A). pCDK1 and caspase3 were detected by western blot and β-actin was the loading control. (D) Increased mitotic catastrophe of B-cell lymphoma upon combined treatment with Ara-C and MK1775. G1XP lymphoma cells were treated as described in (A). Cells undergoing mitotic catastrophe (white arrow) were detected by fluorescent microscopy with DAPI. (E) Increased cell death of Ara-C-induced G2 phase-arrested lymphomas upon MK1775 treatment. CH12 and G1XP lymphoma cells were treated as described in (A). Statistical significance was calculated with one-way ANOVA, Tukey multiple comparison test, *$P \leq 0.05$. (F) G1/S phase-arrested lymphomas upon Ara-C treatment are not sensitive to MK1775 treatment. Ramos, Ly1, Ly7 and DHL-16 lymphomas cells were either untreated or treated with 1 µM Ara-C, 100 nM MK1775, or both for 24 h. Cell numbers were counted and are presented as the percentage of the untreated group. Data are representative results of three independent experiments.

lymphomas and tested whether mitotic entry was affected. Cell cycle analysis showed that Wee1 inhibitor enhanced the mitotic entry of Ara-C-induced G2 phase-arrested lymphomas (Figure 4A,B). Consistently, Wee1 inhibitor reduced the level of pCDK1 (Figure 4C). Furthermore, combined treatment of Ara-C and Wee1 inhibitor induced a higher level of cleaved caspase3 (Figure 4C), promoted more cells to undergo mitotic catastrophe (Figure 4D), and caused more cell death (Figure 4E).

Ara-C treatment resulted in G1 or S phase arrest in other lymphoma lines including DHL-16, Ly1, Ramos, and Ly7 (*Online Supplementary Figure S7*). Intriguingly, Wee1 inhibitor exhibited no effects on the level of mitotic entry (*Online Supplementary Figure S7*), cleaved caspase3 (*Online Supplementary Figure S8*) or cell death (Figure 4F) of the G1 or S phase-arrested lymphomas. Overall, our data suggested that Wee1 inhibitor appears to preferentially promote

mitotic entry, mitotic catastrophe and cell death in G2 phase-arrested lymphomas.

## Newly acquired sensitivity to Wee1 inhibitor in doxorubicin-induced G2-phase-arrested lymphomas

To generalize our findings that Wee1 inhibitor may preferentially affect G2-phase-arrested lymphomas, we treated various lymphoma lines with doxorubicin and found that doxorubicin induced G2 phase-arrest in CH12, Ramos and Ly7 cells but G1 phase-arrest in Ly1 and DHL-16 cells (Figure 5A). Consistently, we showed that combined treatment with doxorubicin and Wee1 inhibitor resulted in more cell death in G2 phase-arrested lymphoma lines compared with doxorubicin alone (Figure 5B). In contrast, the combined treatment had no effects on the survival of non-G2 phase-arrested lymphoma lines (Figure 5C). In line with our findings in Ara-C-treated lymphomas (Figure 4), we found that Wee1 inhibitor also
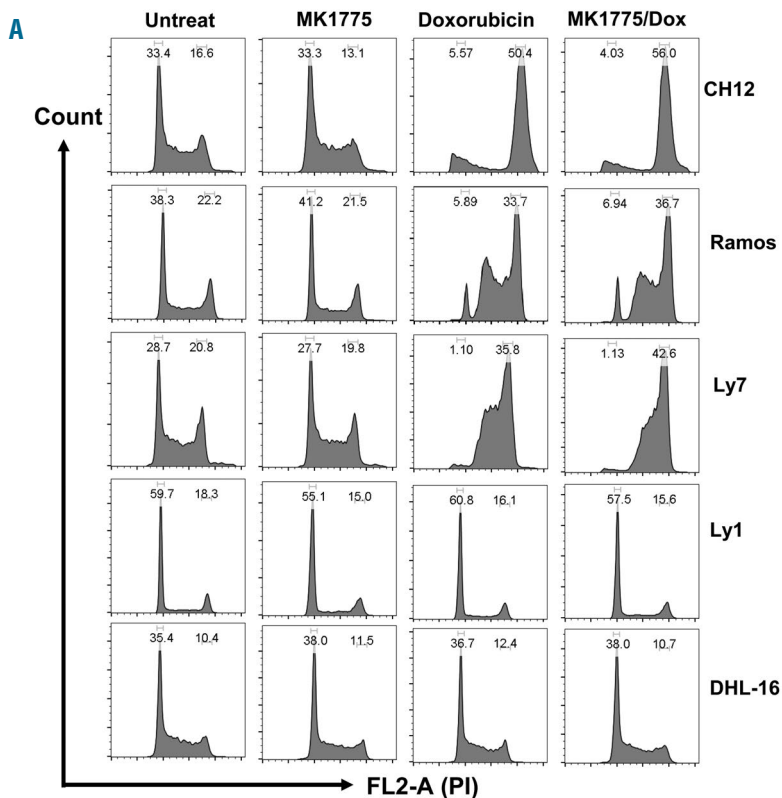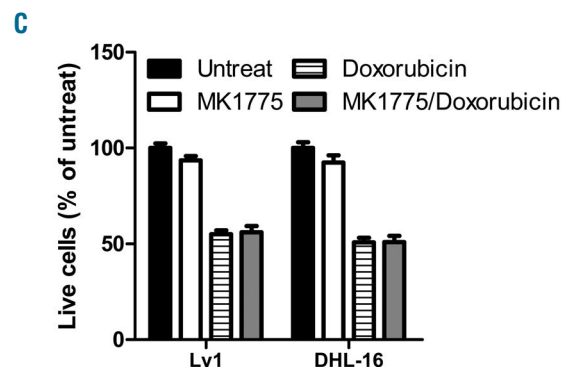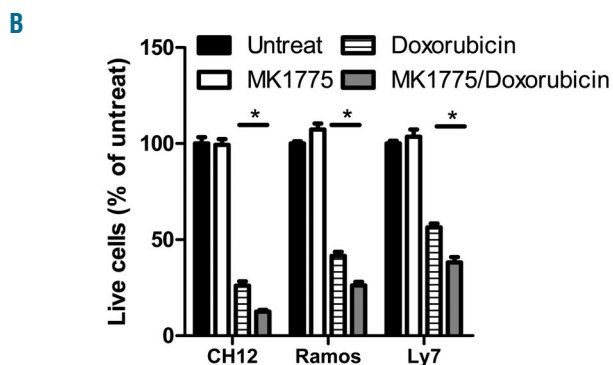


Figure 5. Newly acquired sensitivity to Wee1 inhibitor in doxorubicin-induced G2 phase-arrested lymphoma cells. (A) Cell cycle arrest of various lymphoma lines upon doxorubicin treatment. CH12, Ramos, Ly7, Ly1 and DHL-16 were either untreated or treated with 100 nM doxorubicin, 100 nM MK1775, or both for 24 h. Cells were collected and fixed by 70% ethanol. After propidium iodide (PI) staining, cell cycles were determined by flow cytometry (FL2-A). (B) Increased cell death of doxorubicin-induced G2 phase-arrested lymphomas upon MK1775 treatment. CH12, Ramos and Ly7 lymphomas cells were treated as described in (A). Statistical significance was calculated with one-way analysis of variance, Tukey multiple comparison test, *P≤0.05. (C) Non-G2 phase arrested lymphomas, upon doxorubicin treatment, are not sensitive to MK1775 treatment. Ly1 and DHL-16 lymphoma cells were treated as described in (A). Cell numbers were counted and presented as the percentage of the untreated group. Data are representative results of three independent experiments.

enhanced the mitotic entry of doxorubicin-induced G2 phase-arrested lymphomas (Figure 6A,B), whereas it did not affect the non-G2 phase-arrested lymphomas (Figure 6B). In summary, our data showed that Ramos and Ly7 lymphomas were arrested in G1 or S phase upon Ara-C treatment (*Online Supplementary Figure S7*); however, doxorubicin treatment promoted G2 phase arrest in these two lymphoma lines, which correlated with their newly acquired sensitivity to Wee1 inhibitor.

### Combined Ara-C and Wee1 inhibitor profoundly suppressed tumor growth *in vivo*

To test the therapeutic effects of combined Ara-C and Wee1 inhibitor treatment, we established an *in vivo* transplant model in which G1XP lymphoma cells injected into syngeneic recipient mice develop into secondary B-cell lymphomas (Figure 7A,B). These secondary G1XP lymphomas were initially sensitive to Ara-C treatment, but became unresponsive to continuous Ara-C treatment and relapsed ∼20 days after the initial Ara-C treatment (Figure 7A,B). Wee1 inhibitor alone had no obvious effects on tumor growth or recipient survival compared with the vehicle control (Figure 7A,B). In contrast, combined treatment of Wee1 inhibitor and Ara-C significantly prolonged recipients' survival (Figure 7A) and effectively suppressed tumor growth (Figure 7B). Of note, some of the recipient mice in the Ara-C/MK1775 group died early from unknown causes other than lymphoma. At day 34, tumor size was significantly reduced in the combined treatment group compared with that in the group treated with Ara-C alone (Figure 7C); animals in the groups treated with the vehicle control or Wee1 inhibitor were terminated before this time point due to tumor size exceeding the limit of institutional guidelines. In addition, combined treatment with Ara-C and Wee1 inhibitor attenuated the side effects of Ara-C treatment alone since the weight of recipients was significantly greater in the combined treatment group (Figure 7D). We conclude that, when combined with other chemotherapeutic agents, Wee1 inhibitor may provide a novel therapeutic intervention for B-cell lymphomas that can be arrested in the G2 phase of the cell cycle.

### Discussion

Although Ara-C and doxorubicin have been used in clinics for several decades, it remains incompletely understood how primary B cells or B-cell lymphomas respond to such DNA-damaging agents. In the current study, we employed Ara-C and doxorubicin to treat primary mouse B cells and various B-cell lymphoma lines and present four novel findings: (i) upregulation of cyclinA2 and cyclinB1 appears to be an intrinsically programmed DNA damage response; (ii) Ara-C or doxorubicin induces differential cell cycle arrest in different types of B cells; (iii) Wee1 inhibitor sensitizes G2 phase-arrested B lymphoma cells to Ara-C treatment by inducing premature mitotic entry and mitotic catastrophe. Furthermore, Ara-C-induced G1 phase-arrest can be converted to G2 phase-arrest by doxorubicin treatment in certain B-cell lymphomas (e.g., Ramos cells), which correlates with newly acquired sensitivity to Wee1 inhibitor, and (iv) combined treatment with Ara-C and Wee1 inhibitor profoundly suppressed the tumor growth of transplanted G1XP lymphomas *in vivo*.

We present unexpected findings that, among 1310 pro-

teins included in the SOMAscan assay, only three were differentially expressed between untreated and Ara-C-treated primary B cells, including cyclinB1/A2. However, we failed to identify the functional consequence of cyclinB1/A2 upregulation since double knockdown did not perturb the cell cycle or cause more apoptosis. Perhaps upregulation of these cyclins is indeed a stereotyped universal response to DNA damage, but is only functionally significant in certain types of DNA damage, such as irradiation.[35] Another possibility is that other cyclins may play a compensatory role when cyclins A2 and B1 are knocked down. Our preliminary RNA-seq data did not show the upregulation of cyclinB1/A2 transcripts in the Ara-C-treated group. Hence, we predict that Ara-C treatment may somehow regulate the protein level of cyclinB1/A2, for example, via post-translational modulation of cyclinB1/A2 that leads to high and stabilized protein levels being maintained. Future studies are needed to elucidate the mechanisms that upregulate these cyclins upon Ara-C treatment, independently from blocking the cell cycle.

Previous studies showed that DNA damage often elicits innate immune responses, such as upregulation of NKG2D ligands or interferon responses, in macrophages[36]
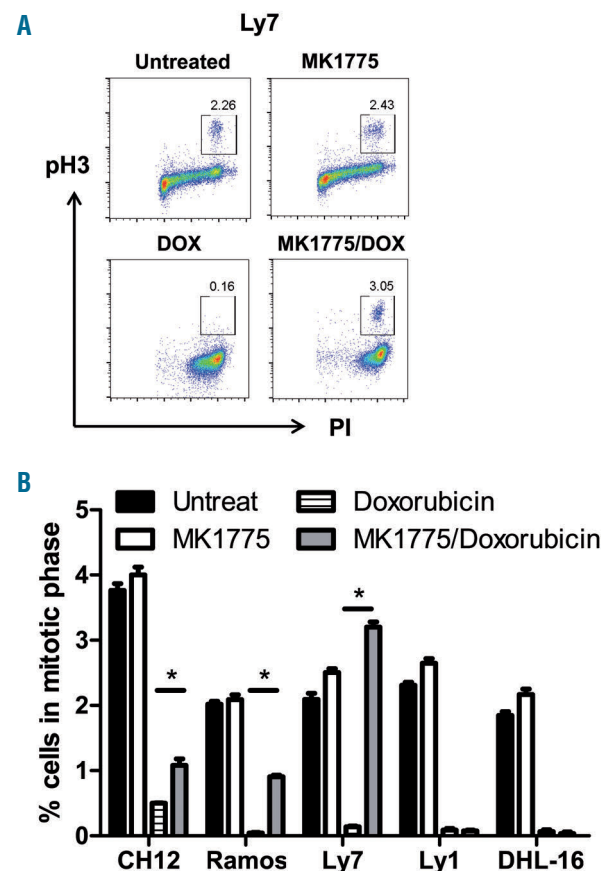
Figure 6. Wee1 inhibitor (MK1775) enhances premature mitotic entry of G2 phase-arrested lymphomas upon treatment with doxorubicin. CH12, Ramos, Ly1, Ly7 and DHL-16 lymphoma cells were treated as described in Figure 5. Cells were collected and fixed by 70% ethanol. After propidium iodide (PI) and pH3 staining, cell cycles were determined by flow cytometry (FL1-H/FL2-A). (A) Representative FACS data of Ly7 lymphoma cells are shown. (B) Statistical analysis of premature mitotic entry in various lymphoma lines. Statistical significance was calculated with ANOVA. Tukey multiple comparison test, *$P \leq 0.05$. Data are representative results of three independent experiments.

or B-cell lymphomas.[37] However, we did not detect upregulation of any factors related to interferon responses in Ara-C-damaged primary B cells. This observation is consistent with previous findings in 3' repair exonuclease 1 (Trex 1) conditional knockout mice.[38] TREX1 is an exonuclease that degrades cytosolic DNA and RNA and one of the unknown substrates of TREX1 can trigger cytoplasmic DNA sensor cyclic GMP–AMP synthase (cGAS). Loss of Trex1 in dendritic cells was sufficient to cause the release of interferon and systemic autoimmunity, whereas, deletion of Trex1 in B cells via CD19Cre did not produce any detectable interferon responses.[38] Taken together, these data suggest that primary B cells appear to tolerate DNA damage with a higher threshold than other types of cells. This phenomenon is likely attributed to the physiological process of programmed generation of double-stranded breaks in primary B cells, namely, class switch recombination and somatic hypermutation. Double-stranded breaks are the essential intermediates of class switch recombination,[39] and recent studies revealed that somatic hypermutation can result in double-stranded break formation in activated B cells.[40] Hence, it may not be surprising that

most non-Hodgkin lymphomas (80-90%) derive from B cells (B-cell non-Hodgkin lymphoma).[41] This higher incidence of B-cell lymphomas is probably attributable to B-cell-specific DNA recombination induced by activation-induced deaminase.[39] While primary B cells do not appear to trigger any immune responses upon DNA damage, this scenario would help to protect DNA-damaged B cells from being attacked by innate immune cells or T cells.

Since Ara-C functions as an S phase-specific chemotherapeutic drug, it would presumably arrest cells in the S phase. We found, however, that different types of B-cell lymphomas were arrested in distinct phases of the cell cycle by Ara-C treatment. The differential cell cycle arrests might depend on the specificity of chemotherapy agents (e.g., Ara-C *versus* doxorubicin), the severity of induced DNA damage and the genetic or epigenetic profiles of different types of B-cell lymphomas. Intriguingly, we found that the cell cycle arrest pattern in certain B-cell lymphomas can be shifted with different chemotherapeutic agents. For instance, Ramos and Ly7 were arrested by Ara-C in the G1 or S phase; however, they could be preferentially arrested in the G2 phase by doxorubicin
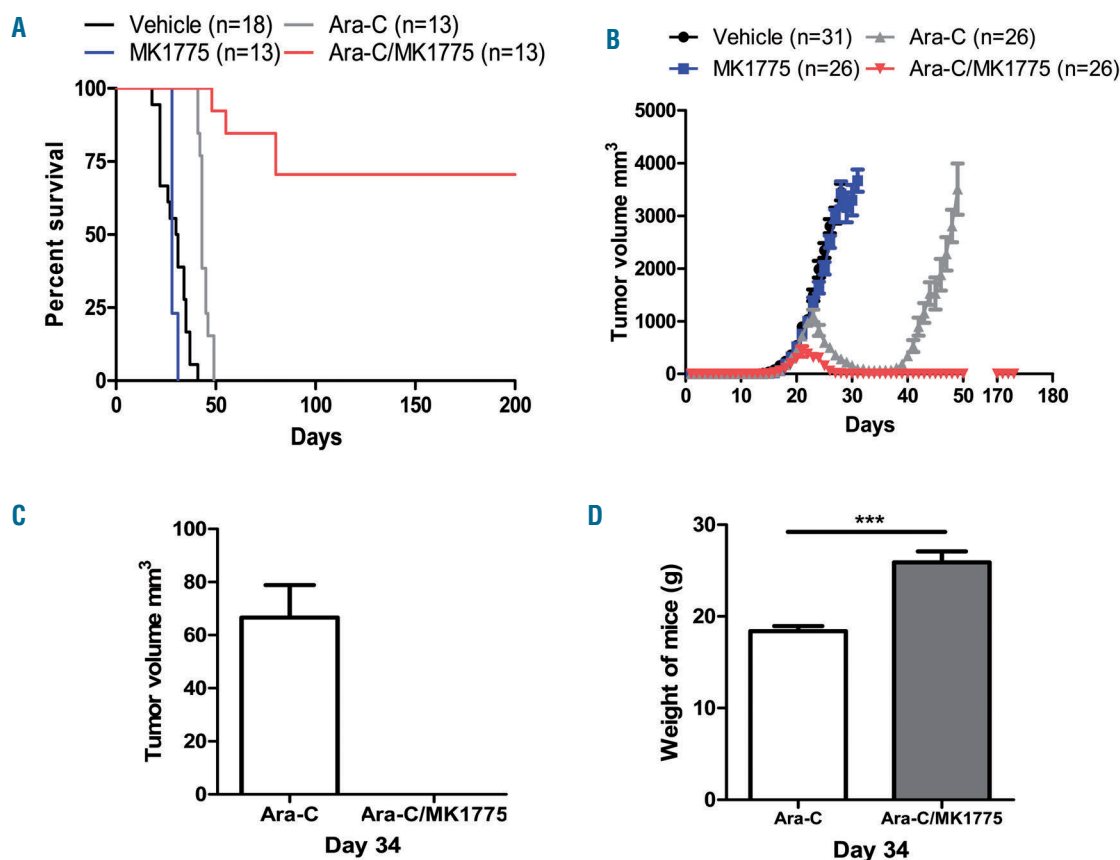


**Figure 7. Combined treatment with Wee1 inhibitor (MK1775) and Ara-C eradicates transplanted G1XP lymphoma and attenuates side effects.** (A) Kaplan-Meier survival curve of recipient mice inoculated with G1XP lymphomas and treated with vehicle (n=18), Ara-C (n=13), MK1775 (n=13) or both (n=13). Data are combined from three independent experiments. When tumor size reached 4000 mm³ or other humane end points were met (e.g., necrotic tumors), mice were euthanized in accordance with institutional guidelines. (B) Combined treatment with Ara-C and MK1775 profoundly suppressed the growth of G1XP lymphomas. Recipients were treated daily with vehicle control or MK1775 from day 21 until termination, or with Ara-C alone from day 21 to day 46, or Ara-C/MK1775 from day 21 to day 28 after tumor inoculation. (C) Combined treatment remarkably reduced the tumor size. The tumor size was monitored for the group treated with Ara-C alone (n=26) *vs.* the Ara-C/MK1775 (n=26) treated group for about 1 week (from day 32 to day 38 after tumor inoculation), whereas the vehicle control and MK1775 groups were already terminated. Data are shown for theday 34 time point. (D) The weight of recipient mice was increased in the combined treatment group. The weight of recipient mice was monitored similarly as described in (C) for the group treated with Ara-C alone (n=13) *vs.* the Ara-C/MK1775 (n=13) group. Data are shown for day 34 time point. Statistical significance was calculated with a T comparison test, ***$P \leq 0.001$.

treatment, which concomitantly occurred with acquired sensitivity to Wee1 inhibitor. We propose that the plasticity of G2 phase-arrest could be exploited for the design of personalized B-cell lymphoma therapy: (i) the cell cycle arrest pattern could be determined upon treatment with different chemotherapeutic agents for each individual patient; (ii) G2 phase-arrest inducing agents could be identified, which could then be combined with Wee1 inhibitor to achieve more effective treatment for the corresponding patient.

Combined treatment with Wee1 inhibitor and Ara-C has been tested for acute myeloid leukemia and T-cell acute lymphocytic leukemia,[14,15] and Wee1 inhibition was also tested in different types of cancers including medulloblastoma and hepatocellular carcinoma;[42-45] however, the underlying mechanisms remain incompletely understood. Mitotic entry is restricted by the phosphorylation of CDK1, and inhibition of pCDK1 could abrogate the G2/M checkpoint and propel G2-phase cells to enter the M phase.[44,46,47] This premature M phase entry would elicit mitotic catastrophe or apoptosis, which has been suggested to act as an effective means of killing cancer cells.[9,13] Previous studies found that Wee1 inhibition promoted G1 or S phase-arrested cells to undergo premature mitotic entry.[16,48] However, our data suggest that only G2 phase-arrested B cell lymphomas were sensitive to combined treatment with Wee1 inhibitor and Ara-C or doxorubicin. In line with *in vitro* data, our *in vivo* transplant G1XP model demonstrated a potential of combined Ara-C and Wee1 inhibitor treatment in eradicating B-cell lymphomas. Prior studies showed that Ara-C treatment activated Chk1,[49] and Chk1 inhibitors synergize with Ara-C in suppressing acute myeloid leukemia.[49,50] Consistently, we found that Ara-C treatment activated Chk1 and Chk2 in most of the B-cell lymphoma lines examined. Overall, these data suggest that cell cycle modulators may enhance the sensitivity of cancer cells to chemotherapeutic agents (e.g., Ara-C or doxorubicin), and that the combinatorial therapy may be more effective. Of note, we observed that a small percentage of recipients died early in the Ara-C/MK1775 group in the absence of lymphoma recurrence, suggesting that the toxic effects of combined Ara-C/MK1775 treatment need to be tested in future studies.

## References

1. Wang WS, Tzeng CH, Chiou TJ, et al. High-dose cytarabine and mitoxantrone as salvage therapy for refractory non-Hodgkin's lymphoma. Jpn J Clin Oncol. 1997;27(3):154-157.
2. Momparler RL. Optimization of cytarabine (ARA-C) therapy for acute myeloid leukemia. Exp Hematol Oncol. 2013;2:20.
3. Vose JM, Link BK, Grossbard ML, et al. Phase II study of rituximab in combination with chop chemotherapy in patients with previously untreated, aggressive non-Hodgkin's lymphoma. J Clin Oncol. 2001;19(2):389-397.
4. Czuczman MS, Grillo-Lopez AJ, White CA, et al. Treatment of patients with low-grade B-cell lymphoma with the combination of chimeric anti-CD20 monoclonal antibody and CHOP chemotherapy. J Clin Oncol. 1999;17(1):268-276.
5. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med. 2002;346(4):235-242.
6. Pfreundschuh M, Trumper L, Osterborg A, et al. CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large-B-cell lymphoma: a randomised controlled trial by the MabThera International Trial (MInT) Group. Lancet Oncol. 2006;7(5):379-391.
7. Tacar O, Sriamornsak P, Dass CR. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. J Pharm Pharmacol. 2013;65(2):157-170.
8. Yang F, Teves SS, Kemp CJ, et al. Doxorubicin, DNA torsion, and chromatin dynamics. Biochim Biophys Acta. 2014;1845(1):84-89.
9. Vitale I, Galluzzi L, Castedo M, et al. Mitotic catastrophe: a mechanism for avoiding genomic instability. Nat Rev Mol Cell Biol. 2011;12(6):385-392.
10. Yuan J, Kramer A, Matthess Y, et al. Stable gene silencing of cyclin B1 in tumor cells increases susceptibility to taxol and leads to growth arrest in vivo. Oncogene. 2006;25(12):1753-1762.
11. Yuan JP, Yan RL, Kramer A, et al. Cyclin B1 depletion inhibits proliferation and induces apoptosis in human tumor cells. Oncogene. 2004;23(34):5843-5852.
12. Soni DV, Sramkoski RM, Lam M, et al. Cyclin B1 is rate limiting but not essential for mitotic entry and progression in mammalian somatic cells. Cell Cycle. 2008;7(9):1285-1300.
13. Denisenko TV, Sorokina IV, Gogvadze V, et al. Mitotic catastrophe and cancer drug resistance: a link that must to be broken. Drug Resist Updat. 2016;24:1-12.
14. Ford JB, Baturin D, Burleson TM, et al. AZD1775 sensitizes T cell acute lymphoblastic leukemia cells to cytarabine by promoting apoptosis over DNA repair. Oncotarget. 2015;6(29):28001-28010.
15. Van Linden AA, Baturin D, Ford JB, et al. Inhibition of Wee1 sensitizes cancer cells to antimetabolite chemotherapeutics in vitro and in vivo, independent of p53 functionality. Mol Cancer Ther. 2013;12(12):2675-2684.
16. Zhou L, Zhang Y, Chen S, et al. A regimen combining the Wee1 inhibitor AZD1775 with HDAC inhibitors targets human acute myeloid leukemia cells harboring various genetic mutations. Leukemia. 2015;29(4):807-818.
17. Chen Z, Getahun A, Chen X, et al. Imbalanced PTEN and PI3K signaling impairs class switch recombination. J Immunol. 2015;195(11):5461-5471.
18. Chen Z, Elos MT, Viboolsittiseri SS, et al. Combined deletion of Xrcc4 and Trp53 in mouse germinal center B cells leads to novel B cell lymphomas with clonal heterogeneity.
19. Chen ZG, Ranganath S, Viboolsittiseri SS, et al. AID-initiated DNA lesions are differentially processed in distinct B cell populations. J Immunol. 2014;193(11):5545-5556.
20. Sampath D, Cortes J, Estrov Z, et al. Pharmacodynamics of cytarabine alone and in combination with 7-hydroxystaurosporine (UCN-01) in AML blasts in vitro and during a clinical trial. Blood. 2006;107(6):2517-2524.
21. Mesa RA, Loegering D, Powell HL, et al. Heat shock protein 90 inhibition sensitizes acute myelogenous leukemia cells to cytarabine. Blood. 2005;106(1):318-327.
22. Nakamura M, Kondo S, Sugai M, et al. High frequency class switching of an IgM+ B lymphoma clone CH12F3 to IgA+ cells. Int Immunol. 1996;8(2):193-201.
23. Linkermann A, Green DR. Necroptosis. N Engl J Med. 2014;370(5):455-465.
24. Dillon CP, Weinlich R, Rodriguez DA, et al. RIPK1 blocks early postnatal lethality mediated by caspase-8 and RIPK3. Cell. 2014;157(5):1189-1202.
25. Orozco S, Yatim N, Werner MR, et al. RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. Cell Death Differ. 2014;21(10):1511-1521.
26. Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, et al. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. Nat Rev Mol Cell Biol. 2014;15(2):135-147.
27. Gold L, Ayers D, Bertino J, et al. Aptamer-based multiplexed proteomic technology for biomarker discovery. PLoS One. 2010;5(12):e15004.
28. Guiraud S, Edwards B, Squire SE, et al. Identification of serum protein biomarkers for utrophin based DMD therapy. Sci Rep. 2017;7:43697.
29. Coenen-Stass AM, McClorey G, Manzano R, et al. Identification of novel, therapy-responsive protein biomarkers in a mouse

J Hematol Oncol. 2016;9:2.

model of Duchenne muscular dystrophy by aptamer-based serum proteomics. Sci Rep. 2015;5:17014.

30. Ersvaer E, Brenner AK, Vetas K, et al. Effects of cytarabine on activation of human T cells - cytarabine has concentration-dependent effects that are modulated both by valproic acid and all-trans retinoic acid. BMC Pharmacol Toxicol. 2015;16:12.

31. Surget S, Khoury MP, Bourdon JC. Uncovering the role of p53 splice variants in human malignancy: a clinical perspective. Onco Targets Ther. 2013;7:57-68.

32. Lane DP. Cancer. p53, guardian of the genome. Nature. 1992;358(6381):15-16.

33. Carr AM, Green MH, Lehmann AR. Checkpoint policing by p53. Nature. 1992;359(6395):486-487.

34. Bieging KT, Mello SS, Attardi LD. Unravelling mechanisms of p53-mediated tumour suppression. Nat Rev Cancer. 2014;14(5):359-370.

35. Porter LA, Singh G, Lee JM. Abundance of cyclin B1 regulates gamma-radiation-induced apoptosis. Blood. 2000;95(8):2645-2650.

36. Hartlova A, Erttmann SF, Raffi FA, et al. DNA damage primes the type I interferon system via the cytosolic DNA sensor STING to promote anti-microbial innate immunity.

Immunity. 2015;42(2):332-343.

37. Lam AR, Le Bert N, Ho SS, et al. RAE1 ligands for the NKG2D receptor are regulated by STING-dependent DNA sensor pathways in lymphoma. Cancer Res. 2014;74(8):2193-2203.

38. Peschke K, Achleitner M, Frenzel K, et al. Loss of trex1 in dendritic cells is sufficient to trigger systemic autoimmunity. J Immunol. 2016;197(6):2157-2166.

39. Chen Z, Wang JH. Generation and repair of AID-initiated DNA lesions in B lymphocytes. Front Med. 2014;8(2):201-216.

40. Yeap LS, Hwang JK, Du Z, et al. Sequence-intrinsic mechanisms that target AID mutational outcomes on antibody genes. Cell. 2015;163(5):1124-1137.

41. Kuppers R. Mechanisms of B-cell lymphoma pathogenesis. Nat Rev Cancer. 2005;5(4): 251-262.

42. Harris PS, Venkataraman S, Alimova I, et al. Integrated genomic analysis identifies the mitotic checkpoint kinase WEE1 as a novel therapeutic target in medulloblastoma. Mol Cancer. 2014;13:72.

43. Kogiso T, Nagahara H, Hashimoto E, et al. Efficient induction of apoptosis by Wee1 kinase inhibition in hepatocellular carcinoma cells. Plos One. 2014;9(6): e100495.

44. Mueller S, Haas-Kogan DA. WEE1 kinase as a target for cancer therapy. J Clin Oncol. 2015;33(30):3485-3487.

45. Pappano WN, Zhang Q, Tucker LA, et al. Genetic inhibition of the atypical kinase Wee1 selectively drives apoptosis of p53 inactive tumor cells. BMC Cancer. 2014;14:430.

46. Elledge SJ. Cell cycle checkpoints: preventing an identity crisis. Science. 1996;274 (5293):1664-1672.

47. van Vugt MA, Medema RH. Getting in and out of mitosis with Polo-like kinase-1. Oncogene. 2005;24(17):2844-2859.

48. Aarts M, Sharpe R, Garcia-Murillas I, et al. Forced mitotic entry of S-phase cells as a therapeutic strategy induced by inhibition of WEE1. Cancer Discov. 2012;2(6):524-539.

49. Schenk EL, Koh BD, Flatten KS, et al. Effects of selective checkpoint kinase 1 inhibition on cytarabine cytotoxicity in acute myelogenous leukemia cells in vitro. Clin Cancer Res. 2012;18(19):5364-5373.

50. Karp JE, Thomas BM, Greer JM, et al. Phase I and pharmacologic trial of cytosine arabinoside with the selective checkpoint 1 inhibitor Sch 900776 in refractory acute leukemias. Clin Cancer Res. 2012;18(24): 6723-6731.