

WEE1 inhibition synergizes with CHOP chemotherapy and radiation therapy through induction of premature mitotic entry and DNA damage in diffuse large B-cell lymphoma

Mathilde R. W. de Jong , Myra Langendonk, Bart Reitsma, Pien Herbers, Monique Lodewijk, Marcel Nijland, Anke van den Berg, Emanuele Ammatuna, Lydia Visser and Tom van Meerten 

Abstract

Background: Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous disease, characterized by high levels of genomic instability and the activation of DNA damage repair pathways. We previously found high expression of the cell cycle regulator WEE1 in DLBCL cell lines. Here, we investigated the combination of the WEE1 inhibitor, AZD1775, with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) and radiation therapy (RT), with the aim of improving first-line treatment.

Methods: Cell viability experiments were performed to determine synergistic combinations. Levels of DNA damage were established using flow cytometry for γ H2AX and protein analysis for DNA damage response proteins CHK1 and CHK2. Flow cytometry analysis for cell cycle and pH3 were performed to determine cell cycle distribution and premature mitotic entry.

Results: Treatment with either RT or CHOP led to enhanced sensitivity to AZD1775 in several DLBCL cell lines. Treatment of cells with AZD1775 induced unscheduled mitotic progression, resulting in abnormal cell cycle distribution in combination with RT or CHOP treatment. In addition, a significant increase in DNA damage was observed compared with CHOP or RT alone. Of the single CHOP components, doxorubicin showed the strongest effect together with AZD1775, reducing viability and increasing DNA damage.

Conclusion: In conclusion, the combination of RT or CHOP with AZD1775 enhances sensitivity to WEE1 inhibition through unscheduled G2/M progression, leading to increased DNA damage. Based on these results, WEE1 inhibition has great potential together with other G2/M arresting or DNA damaging (chemo) therapeutic compounds and should be further explored in clinical trials.

Keywords: AZD1775, CHOP, diffuse large B-cell lymphoma, radiation, WEE1

Received: 6 August 2019; revised manuscript accepted: 9 December 2019.

Introduction

For several decades the first-line treatment for diffuse large B-cell lymphoma (DLBCL) has consisted of the chemotherapy combination of cyclophosphamide, vincristine, doxorubicin and

prednisone (CHOP) together with the anti-CD20 antibody rituximab (R), with or without radiation therapy (RT).¹ Although around 60% of DLBCL patients are cured using R-CHOP, 30–40% will experience disease relapse and 10%

Ther Adv Hematol

2020, Vol. 11: 1–14

DOI: 10.1177/
2040620719898373

© The Author(s), 2020.
Article reuse guidelines:
sagepub.com/journals-
permissions

Correspondence to:
Tom van Meerten
Department of
Hematology, University
of Groningen, University
Medical Center Groningen,
Hanzeplein 1, Groningen
9713 GZ, The Netherlands
t.van.meerten@umcg.nl

**Mathilde Rikje Willemijn
de Jong**
Myra Langendonk
Bart Reitsma
Marcel Nijland
Emanuele Ammatuna
Department of
Hematology, University
of Groningen, University
Medical Center Groningen,
Groningen, The
Netherlands

Pien Herbers
Monique Lodewijk
Anke van den Berg
Lydia Visser
Department of Pathology
and Medical Biology,
University of Groningen,
University Medical Center
Groningen, Groningen, The
Netherlands

of patients develop primary refractory disease,² which has a very poor prognosis. Intensification of chemotherapy has not improved survival and has only led to greater toxicity.³ Furthermore, second generation anti-CD20 antibodies such as ofatumumab, veltuzumab or the third generation anti-CD20 antibody obinutuzumab have all failed to out-perform R in DLBCL.^{4,5} Novel therapies that combine R-CHOP with bortezomib,⁶ lenalidomide⁷ or ibrutinib⁸ have not yet produced the expected improved responses in DLBCL patients. Clearly, there is still an unmet need for improvements to first-line therapies for a significant number of DLBCL patients. New first-line combinations for DLBCL should be based on the targeting of unique and specific DLBCL proteins and should act synergistically with R-CHOP.

We recently identified WEE1 as a promising target in DLBCL and demonstrated its efficacy as a druggable target in combination with rituximab.⁹ WEE1 is a cell cycle regulator that inhibits G2/M transition by blocking CDC2/CDK1 activity through phosphorylation at Tyr15. In the event of DNA damage, WEE1 is activated by CHK1 to allow DNA damage repair before the cell transitions into mitosis.¹⁰ Many tumour cells rely on this cell cycle checkpoint for repair because they have lost the ability to repair DNA damage at the G1/S phase as a result of TP53 abnormalities (deletions or mutations) or mutations in other cell cycle controlling genes, often resulting in diminished activity of the CDK inhibitor p21.¹¹ In DLBCL, aberrant and off-target activity of activation-induced cytidine deaminase induces oncogenic mutations and chromosomal translocations,¹² causing genetic instability¹³ and activation of DNA damage repair pathways.¹⁴ In this setting, targeting WEE1 could prevent proper initiation of the G2/M checkpoint, leading to accumulation of DNA damage and mitotic catastrophe. Inhibition of WEE1 using AZD1775 has been shown to be effective in many different tumour types, especially in tumours with compromised DNA damage response pathways such as TP53-deficient ovarian adenocarcinoma tumour cells.¹⁵ Since WEE1 inhibition prevents adequate DNA damage repair, targeting WEE1 in cells with an already compromised DNA damage repair pathway or inducing additional DNA damage through genotoxic compounds would seem a rational application for WEE1 inhibition in DLBCL treatment.

Here, we investigated the effect of the WEE1 inhibitor AZD1775 combined with standard RT or CHOP therapy. We observed a synergistic effect of AZD1775 with both RT and CHOP, as treatment of DLBCL cells with RT or CHOP led to increased expression of the DNA damage response proteins CHK1 and CHK2, increased levels of the DNA damage marker γ H2AX and increased levels of premature mitotic entry. Together, these findings indicate that blocking WEE1, in combination with RT or CHOP, limits the time necessary for DNA repair due to unscheduled mitosis, increases DNA damage and ultimately results in enhanced cell death. These data indicate the potential of WEE1 inhibition in combination with first-line RT and CHOP therapy for DLBCL patients and highlight the clinical potential of AZD1775 in DLBCL treatment.

Materials and methods

Cell lines and culture conditions

The DLBCL cell lines U-2932, SUDHL-2, SUDHL-4 and SC-1 were cultured in suspension in Roswell Park Memorial Institute medium 1640 (RPMI 1640; Lonza BioWhittaker, Walkersville, MD, USA) with 10% foetal bovine serum (FBS; HyClone Thermo Scientific, Waltham, MA, USA), 1% penicillin-streptomycin (PS; Lonza BioWhittaker) and 1% glutamine (Lonza BioWhittaker). The DLBCL cell lines OCILY3, SUDHL-5, SUDHL-6 and SUDHL-10 were cultured in suspension in RPMI 1640 with 20% FBS, 1% PS and 1% glutamine. All cell lines were cultured at 37°C with 5% CO₂ in a humidified atmosphere. The identity of our cell lines was regularly checked using short tandem repeat typing and their TP53 gene status was determined by sequencing exons 1–10.

Compounds and radiation

The WEE1 inhibitor AZD1775 was acquired from Selleckchem (No.S1525, Houston, TX, USA). RT was performed at a dosage from 2 to 20 Gy using a Cesium-137 source-662 keV photons (IBL 637, Cis Bio International, Gif-sur-Yvette, France) and metabolic activity of cells was measured after 72 h. CHOP included cyclophosphamide (University Medical Center Groningen [UMCG] pharmacy), doxorubicin (No.S1208, Selleckchem), vincristine (UMCG pharmacy) and prednisolone (No.S1737,

Selleckchem), in a composition set at the clinical ratio of 83/5.5/0.16/11.1, respectively.¹⁶

Metabolic activity

Metabolic activity of cells was measured after 72 h treatment of 0.4×10^6 cells/ml. Cells were incubated with 10 μ l resazurin (5% final concentration, AlamarBlue, Thermo Fisher Scientific, Waltham, MA, USA) for 9 h prior to read-out (Varioskan, excitation 560 nm, emission 590 nm). Experiments were performed five times.

Flow cytometry: cell cycle, γ H2AX and pH3 with DNA content

For cell cycle analysis, 0.2×10^6 cells/ml were treated for the indicated time points, washed with 1% bovine serum albumin/phosphate-buffered saline (PBS) and resuspended in solution containing 0.1% sodium citrate (A0158348, Merck, Kenilworth, NJ, USA), 0.01% propidium iodide (P4170, Sigma-Aldrich, St. Louis, MO, USA), 0.002% RNase A (R4875, Sigma-Aldrich) and 0.3% Triton X100 (T9284, Sigma-Aldrich). Samples were processed on a BD FACSCalibur 2 and analysed with ModFit LT (Verity Software House). Experiments were performed three times.

For γ H2AX analysis, 0.2×10^6 cells/ml were treated for the indicated time points and then stained with mouse anti- γ H2AX-AlexaFluor-647 (clone 2F3, #613408, BioLegend) and propidium iodide solution (P4170, Sigma) according to the protocol provided with the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher, #00-5523-00). Samples were processed on a MACSQuant and the data were analysed using Kaluza 1.5 analysis software (Beckman). Experiments were performed three times.

For pH3 analysis, 0.2×10^6 cells/ml were treated for the indicated time points and stained with mouse-anti-pH3-AlexaFluor-647 (clone 11D8, #650806, Biolegend) and propidium iodide solution (P4170, Sigma) according to the protocol provided with the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher, #00-5523-00). Samples were processed on a MACSQuant and data were analysed using Kaluza 1.5 analysis software (Beckman). Premature mitotic cells were identified as pH3 positive cells measured in S-phase

instead of in G2/M phase. Experiments were performed three times.

Western blot

Cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer (50mM Tris, 150mM NaCl, 2.5mM Na2EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS in dH₂O) with 1 mM phenylmethanesulphonyl fluoride for 30–45 min on ice. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (#23227; Thermo Scientific, Waltham MA, USA). Samples were loaded at 40 μ g per lane and electrophoresis and blotting was performed according to standard protocols. Staining with primary antibodies for anti-WEE1 (1:200, sc-5285 (B11), Santa Cruz Biotechnology, Dallas TX, USA), anti-phospho-CDC2 (Tyr15) (10A11) (1:1000, #4539, Cell Signaling Technology, Danvers, MA, USA), anti-phospho-Histone H2AX (Ser139) (1:1000, clone JBW301, Merck Millipore, Temecula, CA, USA) and GAPDH (1:10,000; NovusBio) was done overnight at 4°C.

Statistical analysis

Data were analysed using Graphpad PRISM (version 5.0) software and tested for significant differences using a paired *t*-test. Bubble graphs were plotted, with the position of each bubble on the y-axis representing an IC₅₀ value and the size representing the calculated factor differences *versus* the control. The size of each bubble is an arbitrary unit, determined by the factor and fold change between the combination IC50 (AZD + treatment) *versus* AZD alone (=1). This is calculated with the formula: 1 (factor of AZD alone) * IC50 AZD + treatment ÷ IC50 AZD alone. If the IC50 value could not be calculated the value is marked with 'not available'. Synergism of combination therapies was calculated using the method of combination sub-thresholding, which calculates the difference between the expected effect (E) of the combination therapy compared with the observed effect (O) of the combination therapy and tested for significant differences using a paired *t*-test. Corresponding significant *p*-values were colour coded to represent additive combinations in white (E = O), synergistic combinations in green (E < O) and antagonistic combinations in red (E > O). * indicates *p* ≤ 0.05, ** indicates *p* ≤ 0.01 and *** indicates *p* ≤ 0.001.

Results

Radiation and CHOP therapy enhance sensitivity to AZD1775 in DLBCL cell lines

RT and CHOP chemotherapy are part of the standard treatment for DLBCL and both induce high levels of DNA damage thereby causing cell death. Efficient DNA damage repair and survival and/or resistance require both activation of the DNA damage response and cell cycle arrest. We hypothesized that WEE1 inhibition together with RT and CHOP prevents cell cycle arrest and allows DNA damage to accumulate against a background of repair inhibition, resulting in enhanced cell killing. Titration of AZD1775 in eight DLBCL cell lines revealed a wide range of sensitivities to AZD1775, with a minimum IC_{50} value of 357 nM for OCI-LY3 to a maximum IC_{50} value of 1835 nM for SUDHL-4, values that are comparable with or less than the clinical C_{max} serum concentration for AZD1775 of 1650 nmol/L in solid tumours¹⁷ (Supplemental 1a). The combination of AZD1775 with RT showed a dose-dependent decrease in the IC_{50} values of all cell lines, thereby enhancing sensitivity to AZD1775. The combination of RT and AZD1775 was synergistic in six out of eight cell lines (Figure 1a and Supplemental 2a). Combination of AZD1775 with 4 Gy radiation resulted in a 5.5-fold increased sensitivity to AZD1775 (IC_{50} decrease from 357 nM to 65 nM) in OCI-LY3. The combination of AZD1775 together with CHOP chemotherapy gave similar results, with a dose-dependent decrease in the IC_{50} values of all cell lines, resulting in synergism in seven out of eight cell lines (Figure 1b and Supplemental 2b). An up to 6-fold enhanced sensitivity to AZD1775 was observed in SUDHL-10 (IC_{50} decrease from 608 nM to 100 nM) (Figure 1b). Analysis of the *TP53* status of our cell lines revealed no correlation between *TP53* mutation status and IC_{50} values for AZD1775 ($p=0.2500$) (Supplemental 1b), CHOP ($p=0.5446$) (Supplemental 1c) or RT ($p=0.1876$) (Supplemental 1d) nor the response to combination therapy. In conclusion, these results demonstrate the great potential of the WEE1 inhibitor AZD1775 in combination with CHOP and RT as a first-line standard therapy in DLBCL.

AZD1775 with RT or CHOP therapy activates the DNA damage response

Next, we investigated if the synergistic effect of WEE1 inhibition together with DNA damaging agents (RT or CHOP) was a result of increased DNA damage in the representative cell lines

SUDHL-5, SUDHL-6 and SUDHL-10. Flow cytometry analysis of the DNA damage marker γ H2AX showed a moderate increase in γ H2AX positive cells (3–13%) after treatment with AZD1775 (Figure 2A–B). Similarly, radiation alone at the suboptimal dose of 2 and 4 Gy induced little DNA damage (5–11% γ H2AX positive cells) (Figure 2a). However, the combination of RT with AZD1775 led to a dose-dependent increase of γ H2AX positive cells. In SUDHL-6, 4 Gy radiation produced 10% γ H2AX positive cells, increasing to 34% when combined with 500 nM AZD1775 ($p=0.02$) (Figure 2a). Treatment with CHOP (Figure 2b) at a suboptimal dose of 0.1 μ g/ml induced minimal DNA damage (5–7% γ H2AX positive cells) in all cell lines, whereas 1 μ g/ml induced significant levels of DNA damage (39–54% γ H2AX positive cells) in DLBCL cell lines. The addition of 500 nM AZD1775 to 0.1 μ g/ml CHOP significantly increased γ H2AX positive cells (from 7% to 40%; $p=0.0107$) in both SUDHL-5 and SUDHL-6 (5–16%; $p=0.0261$). At the CHOP dosage of 1 μ g/ml, addition of AZD1775 did not further increase γ H2AX positive cells, indicating that cells had reached their maximum potential for DNA damage. A dose-dependent increase in γ H2AX positive cells was observed for the combination of CHOP with AZD1775 in SUDHL-10, which was most prominent in the range 500 nM AZD1775 to 1 μ g/ml CHOP, producing a significant increase in γ H2AX positive cells (from 1% to 40%; $p=0.0003$). These findings demonstrate that the combination of a suboptimal dose of AZD1775 together with a suboptimal dose of either RT or CHOP leads to a strongly increased level of DNA damage.

Following up these results, we next performed protein analysis of the DDR kinases, checkpoint kinase 1 (CHK1) and checkpoint kinase 2 (CHK2), which are activated in response to single-stranded DNA breaks and double-stranded DNA breaks, respectively.¹⁸ Treatment of the cell lines SUDHL-5 (Figure 3a), SUDHL-6 (Figure 3b) and SUDHL-10 (Figure 3c) with AZD1775 or RT alone for 3 h increased phospho-CHK1 levels (Ser345). These effects were more prominent when RT was combined with AZD1775. Although no induction of phospho-CHK1 was observed in response to CHOP monotherapy, an increase was observed combination with AZD1775. In SUDHL-5, protein levels of phospho-CHK2 (Thr68) were increased

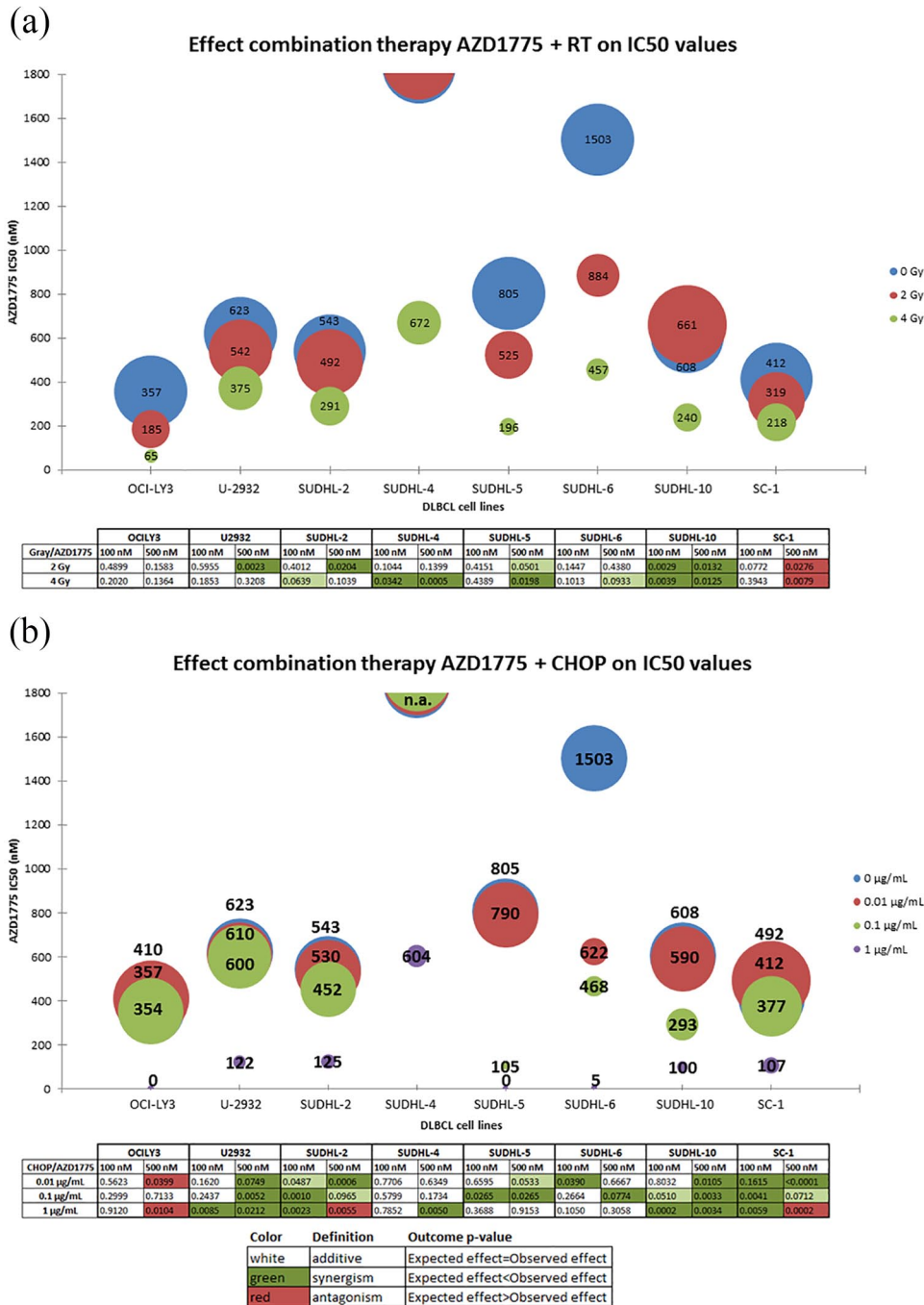


Figure 1. AZD1775 combination therapy with radiation therapy (RT) or CHOP in DLBCL cell lines. (a) IC₅₀ values are plotted for DLBCL cell lines treated with AZD1775 alone (blue), together with either 2 gray (red) or 4 gray (green) ionizing radiation; (b) IC₅₀ values are plotted for DLBCL cell lines treated with AZD1775 alone (blue), together with 0.01 µg/mL CHOP (red), with 0.1 µg/mL CHOP (green) or with 1 µg/mL CHOP (purple); (c) IC₅₀ values were determined from metabolic activity data measured after 72 hours of treatment using resazurin. Data were normalized to the control and plotted as the mean IC₅₀ of n=3; (d) Synergy of each combination was calculated as the difference between the expected effect; (e) compared to the observed effect (O) of the combination therapy, with a corresponding (significant) p-value, analysed for significance using a one-sample T-test. Colours represent additive in white (E=0), synergy in green (E<0) and antagonism in red (E>0).

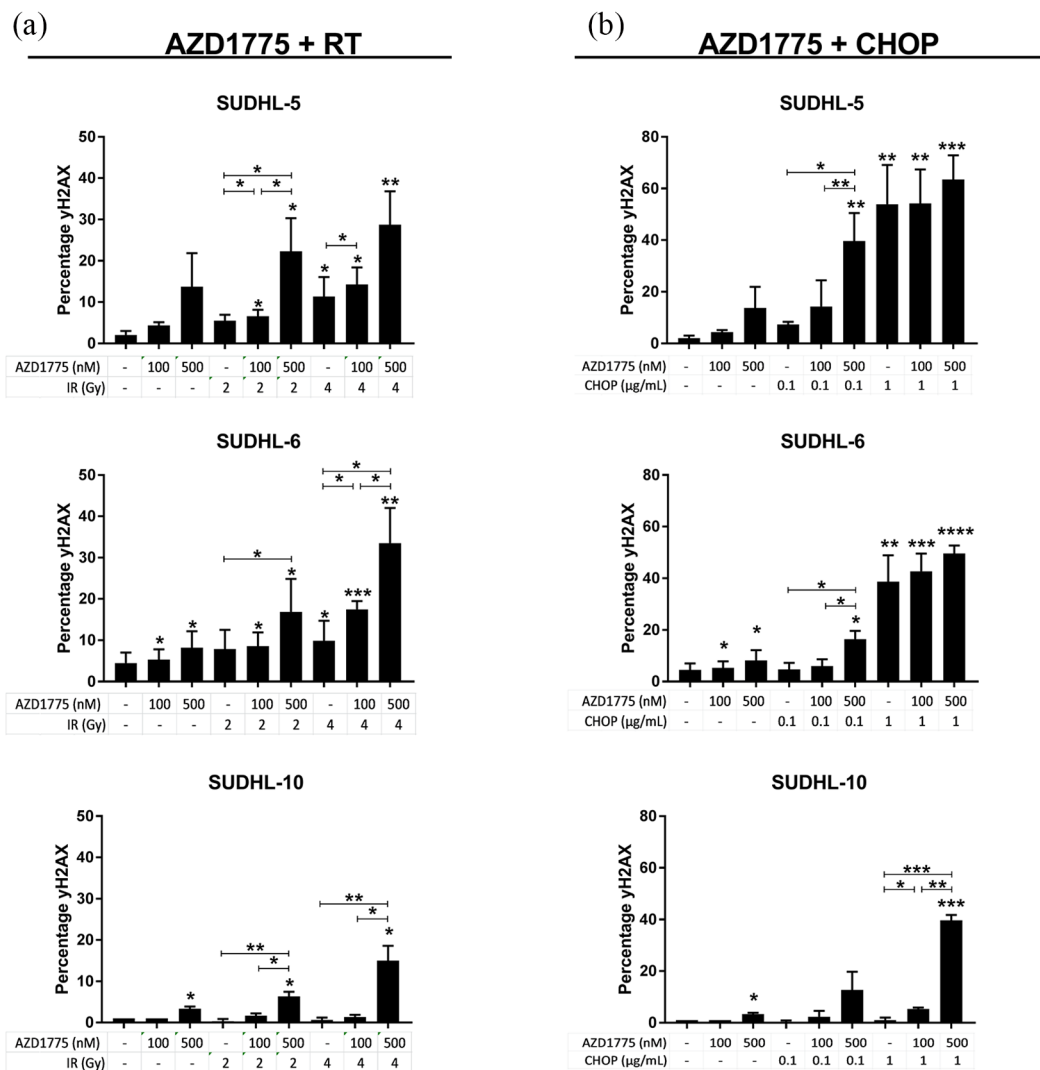


Figure 2. DNA damage analysis of AZD1775 combined with RT or CHOP. (a) Analysis of γ H2AX positive cells in the cell lines SUDHL-5, SUDHL-6 and SUDHL-10 treated for 24 hours with 100 nM and 500 nM AZD1775 combined with 2 gray or 4 gray radiation therapy (RT); (b) Analysis of γ H2AX positive cells in SUDHL-5, SUDHL-6 and SUDHL-10 treated for 24 hours with 100 nM and 500 nM AZD1775 combined with 0.1 or 1 μ g/mL CHOP. Data are plotted as the mean \pm SD of n=3. Statistical analysis was performed using a one-sample T-test (* $p \leq 0.05$) (** $p \leq 0.01$) (***) $p \leq 0.001$).

(Figure 3a) upon treatment with RT and CHOP monotherapy, however no further increase was observed by addition of AZD1775. In the cell line SUDHL-6 (Figure 3b) treatment with RT strongly induced phospho-CHK2, whereas little effect was observed by CHOP chemotherapy. Addition of AZD1775 had little additional effect. In cell line SUDHL-10 (Figure 3c), only a mild phospho-CHK2 induction was observed upon RT or CHOP treatment, whereas addition of AZD1775 to RT strongly enhanced phospho-CHK2 expression.

In all three cell lines mentioned above, treatment with AZD1775 led to a significant reduction in phospho-CDC2 (Tyr15; pCDC2) levels, the downstream target of WEE1, indicating that WEE1 kinase activity was reduced but without an effect on WEE1 protein levels. In conclusion, these data show activation of both CHK1 and CHK2 DDR pathways, indicating that the combination of AZD1775 with RT or CHOP induces harmful DNA damage. Combined protein and γ H2AX results show that while monotherapy with AZD1775, radiation or CHOP can activate the

DDR (phospho-CHK1 and phospho-CHK2) in response to treatment, the combination of RT or CHOP with AZD1775 is much more effective in inducing high levels of DNA damage.

AZD1775 abrogates mitotic arrest and induces premature mitotic entry

We next studied the effect of AZD1775, RT or CHOP monotherapy and combination therapy on cell cycle distribution and premature mitotic entry, measured as phospho-H3 (Ser10) positive cells in S-phase (Figure 4). Cell cycle analysis in the cell lines SUDHL-5, SUDHL-6 and SUDHL-10 showed that monotherapy with radiation induced a significant dose-dependent increase in the percentage of G2/M phase cells, which was most prominent in SUDHL-10 (with an increase from 21% to 80%; $p=0.0047$) after 4 Gy radiation (Figure 4a). The addition of AZD1775 induced a dose-dependent reduction in the percentage of G2/M phase cells (decreased from 80% to 46%; $p=0.0025$), an effect also observed in SUDHL-5 and SUDHL-6 (Figure 4a). These cell cycle data were linked to the data on phospho-H3 positive cells, which normally occurs during mitosis, but can be observed during S-phase in the situation of premature mitotic entry. Analysis of phospho-H3 positive cells in S-phase revealed that AZD1775 treatment significantly increased premature mitosis in SUDHL-5 ($p=0.0132$), and SUDHL-10 ($p=0.0008$) and a trend for SUDHL-6 ($p=0.0842$) compared with control cells, while no effect was observed for RT monotherapy (Figure 4b). Moreover, levels of premature mitotic entry were increased in the cell lines SUDHL-5 and SUDHL-6 when AZD1775 was combined with RT, although the changes were not statistically significant. Together, these data indicate that addition of AZD1775 to RT reduces the G2/M arrest induced by RT, by allowing these cells to re-enter the cell cycle, but at the same time are likely over-stimulating cell cycle progression by facilitating premature mitotic entry. Cell cycle analysis of CHOP monotherapy showed little effect for CHOP at 0.1 $\mu\text{g}/\text{ml}$, whereas CHOP treatment at 1 $\mu\text{g}/\text{ml}$ significantly increased the percentage of G2/M phase cells in SUDHL-5 ($p=0.0040$), SUDHL-6 ($p=0.0349$) and SUDHL-10 ($p=0.0237$) (Figure 4c). However, no rescue of the CHOP-induced G2/M arrest was observed when CHOP was combined with AZD1775, like was

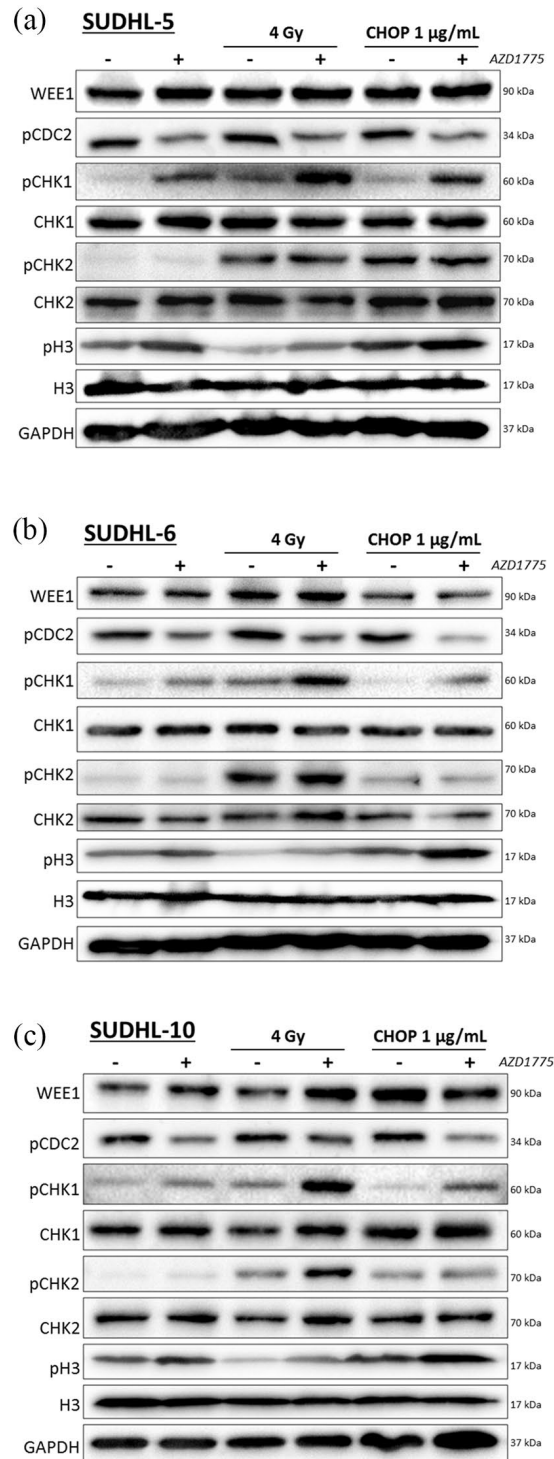


Figure 3. Protein analysis of the DNA damage response after treatment with AZD1775 in combination with RT or CHOP. Western blot analysis of WEE1, pCDC2 (Tyr15), pCHK1 (Ser345), CHK1, pCHK2 (Thr68), CHK2, pH3 (Ser10), H3 and GAPDH protein levels in the (a) HL-5; b) DHL-6, and (c) L-10 cell lines treated with 500 nM AZD1775, 4 gray RT and 1 $\mu\text{g}/\text{ml}$ CHOP for 3 hours.

AZD1775 + CHOP

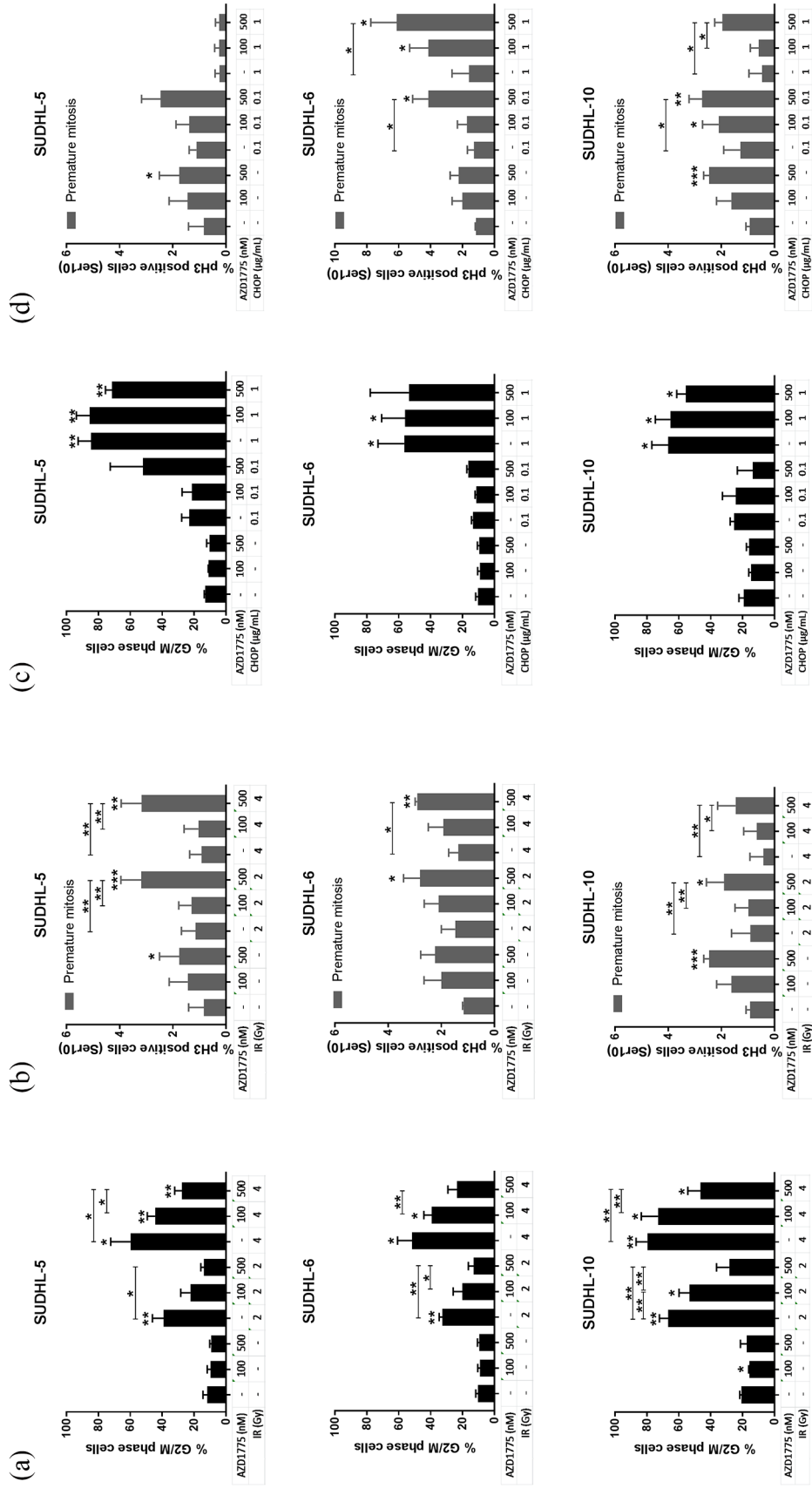


Figure 4. Cell cycle and pH3 analysis of AZD1775 combined with RT or CHOP. (a) Percentage of G2/M phase cells treated for 24 hours with 100 nM and 500 nM AZD1776 combined with 2 gray or 4 gray radiation therapy (RT) in the cell lines SUDHL-5, SUDHL-6 and SUDHL-10; (b) Percentage of phospho-H3 (Ser10) positive cells in S-phase treated for 24 hours with 100 nM and 500 nM AZD1776 combined with 2 gray or 4 gray radiation therapy (RT) in SUDHL-5, SUDHL-6 and SUDHL-10; (c) Percentage of G2/M phase cells treated for 24 hours with 100 nM and 500 nM AZD1776 combined with 0.1 µg/mL or 1 µg/mL CHOP in SUDHL-5, SUDHL-6 and SUDHL-10. D) Percentage of phospho-H3 (Ser10) positive cells treated for 24 hours with 100 nM and 500 nM AZD1776 combined with 0.1 µg/mL or 1 µg/mL CHOP in SUDHL-5, SUDHL-6 and SUDHL-10. Data are plotted as the mean ± SD of n=3. Statistical analysis was performed using a one-sample T-test (*p≤0.05) (**p≤0.01) (***)p≤0.001).

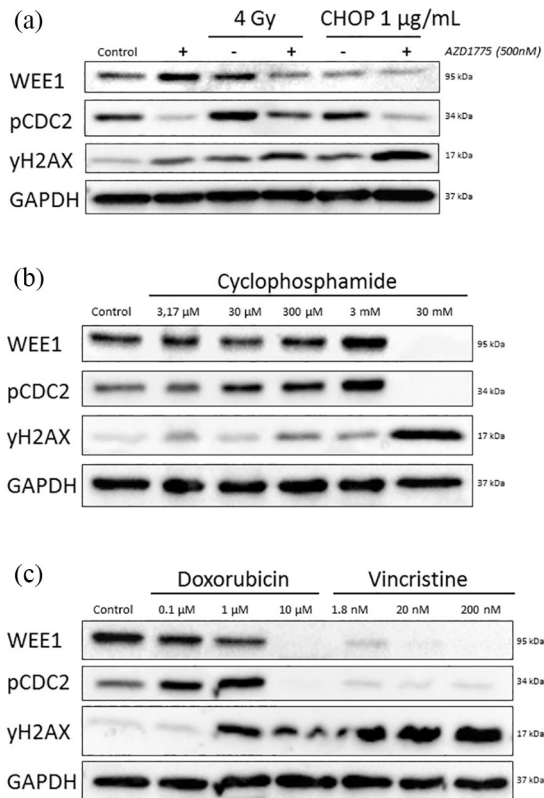


Figure 5. Figure 5. WEE1 protein activity analysis after treatment with radiation, CHOP or doxorubicin, vincristine and cyclophosphamide monotherapy. (a) Western blot analysis of WEE1, pCDC2 (Tyr15), γ H2AX and GAPDH protein levels in SUDHL-10 treated with AZD1775 in combination with 4 gray RT or 1 μ g/mL CHOP for 24 hours; (b) Western blot analysis of WEE1, pCDC2 (Tyr15) and γ H2AX in SUDHL-10 treated with an increasing concentration of doxorubicin and vincristine for 24 hours; (c) Western blot analysis of WEE1, pCDC2 (Tyr15) and γ H2AX in SUDHL-10 treated with an increasing concentration of cyclophosphamide for 24 hours. SUDHL-10 cells were treated with a 10-fold increasing concentration of each compound, the lowest concentration of which [0.1 μ M doxorubicin, 1.8 nM vincristine and 3.17 μ M cyclophosphamide] was used in the 1 μ g/mL CHOP treatment.

previously observed for the combination of AZD1775 with RT (Figure 4a). Analysis of premature positive phospho-H3 cells revealed a dose-dependent increase in premature mitotic entry when cells were treated with AZD1775 or when AZD1775 was combined with CHOP, while no effect was observed for CHOP alone (Figure 4d). In SUDHL-5, a strong decrease was observed in phospho-H3 cells treated with 1 μ g/ml CHOP, which was caused by a strong induction of DNA damage (Figure 2b) and cell death (Figure 1). Taken together, these data indicate

that RT likely induces G2/M phase arrest to allow DNA repair, which is abrogated by addition of AZD1775, resulting in premature mitotic entry and increased DNA damage. A different effect can be observed for the combination of AZD1775 with CHOP, in which the combination of CHOP with AZD1775 does not rescue the CHOP-induced G2/M phase arrest. This difference is likely caused by the multiple detrimental effect of the CHOP compounds attacking the tubulin network and/or the DNA structure, resulting in structural damage and irreversible cell cycle arrest, accompanied by high levels of DNA damage.

Radiation, cyclophosphamide and doxorubicin enhance WEE1 protein expression

To further investigate the effect of RT or CHOP treatment, with or without AZD1775, on WEE1 kinase activity, we performed protein analysis in the cell line SUDHL-10 treated with the respective CHOP compounds for 24 h (Figure 5a–b). Protein analysis showed that RT led to an increased pCDC2 level, indicating increased WEE1 activity, which was subsequently reduced to control levels on addition of AZD1775 (Figure 5a). This analysis suggests that upregulated WEE1 activity acts to control RT-induced DNA damage, as observed by the increased γ H2AX levels measured by flow cytometry (Figure 2a) and DDR activation by western blot (Figure 3a). No changes in pCDC2 levels were observed upon CHOP monotherapy treatment (Figure 5a). We next investigated the effect of individual CHOP components on WEE1 protein activity, treating cells with each distinct component at the concentration used in 1 μ g/ml CHOP or at a concentration that was 10/100/1000-fold higher. The individual compounds present in 1 μ g/ml CHOP were 0.1 μ M doxorubicin, 1.8 nM vincristine and 3.17 μ M cyclophosphamide, all of which are below clinical C_{max} dosages.¹⁹ Cyclophosphamide monotherapy at low non-toxic dosages increased WEE1, pCDC2 and γ H2AX protein levels in a dose-dependent manner, while toxic treatment at 30 mM reduced both WEE1 and pCDC2 protein levels (Figure 5c). Similarly, doxorubicin monotherapy induced a strong dose-dependent increase of pCDC2 and γ H2AX levels compared with control cells, which was absent in cells treated with a toxic concentration of 10 μ M (Figure 5c). Unlike cyclophosphamide and doxorubicin,

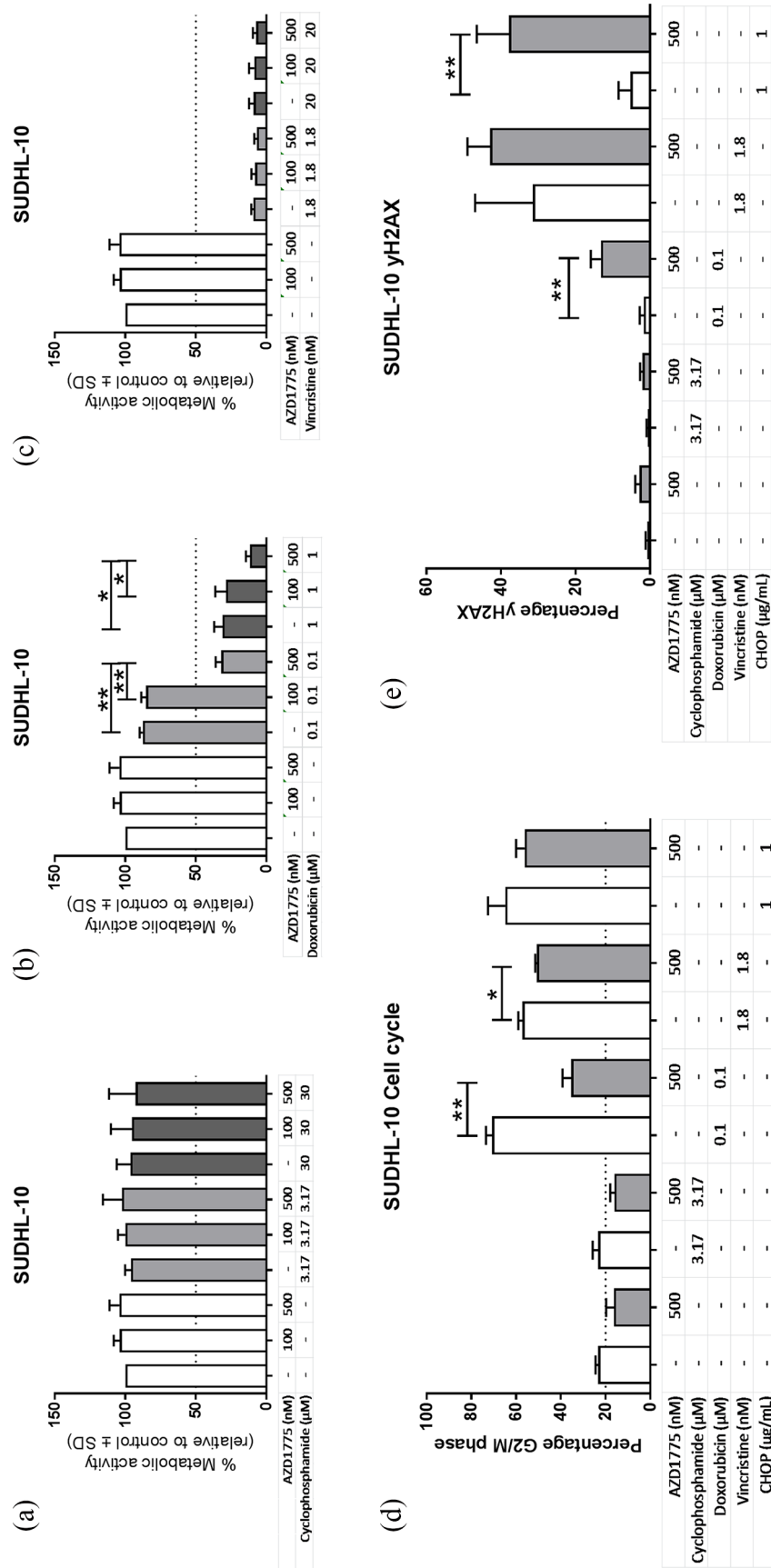


Figure 6. Combination therapy of AZD1775 and single CHOP compounds. (a) Metabolic activity determined by resazurin analysis of SUDHL-10 treated for 72 hours with cyclophosphamide and AZD1775. (b) Metabolic activity by resazurin analysis of SUDHL-10 treated for 72 hours with doxorubicin and AZD1775; (c) Metabolic activity by resazurin analysis of SUDHL-10 treated for 72 hours with vincristine and AZD1775; (d) G2/M phase flow cytometry analysis of SUDHL-10 treated with AZD1775 and cyclophosphamide, doxorubicin or vincristine for 24 hours; (e) γH2AX flow cytometry analysis of SUDHL-10 treated with AZD1775 and cyclophosphamide, doxorubicin or vincristine for 24 hours. Data were normalized to the control and plotted as the mean ± SD of n=3. Statistical analysis was performed with a one-sample T-test (*p≤0.05) (**p≤0.01) (***)p≤0.001 (N=3).

vincristine monotherapy actually reduced both pCDC2 and WEE1 protein levels at the low 1.8 nM dose, while upregulating γ H2AX protein levels (Figure 5c). These data show that although cyclophosphamide and doxorubicin are able to increase WEE1 activity, these changes are likely suppressed by the presence of vincristine in CHOP, thereby counteracting the upregulation of the WEE1 protein.

Doxorubicin and vincristine enhance AZD1775-induced cell killing

Pursuing the finding that certain CHOP components can upregulate WEE1 protein expression, we next determined if single CHOP components (cyclophosphamide, doxorubicin or vincristine) complement AZD1775 treatment in the SUDHL-10 cell line. Cyclophosphamide treatment alone or in combination with AZD1775 had no effect on metabolic activity, indicating that the concentrations used were too low to affect viability (Figure 6a). Doxorubicin treatment combined with 500 nM AZD1775 significantly reduced metabolic activity (from 88% to 32%; $p=0.0023$), indicating that upregulation of WEE1 protein by doxorubicin enhanced sensitivity to AZD1775 (Figure 6c). Single agent treatment with vincristine decreased metabolic activity by 91% compared with control, with no additional effect of AZD1775 (Figure 6b).

Analysis of the cell cycle showed that both doxorubicin and vincristine monotherapy increased the percentage of G2/M phase cells, while no effect was observed for cyclophosphamide (Figure 6d). Similar to previous results, addition of AZD1775 decreased G2/M percentages, indicating premature mitotic entry.

Analysis of γ H2AX showed that doxorubicin monotherapy induced minimal γ H2AX positive cells, but addition of AZD1775 significantly increased γ H2AX from 2% to 13% ($p=0.0083$) (Figure 6e). Vincristine was particularly effective in inducing DNA damage, especially in combination with AZD1775 (γ H2AX increased from 31% to 43%). Remarkably, the DNA damaging effect of vincristine was lost in CHOP chemotherapy, since CHOP only induced 5% γ H2AX positive cells compared with 31% γ H2AX positive cells induced by vincristine. Nevertheless, the combination of AZD1775 with CHOP resulted in a significant increase in γ H2AX levels (5–38% with

$p=0.0092$), similar to the levels achieved by vincristine alone (31%) or vincristine combined with AZD1775 (43%). Taken together, these results demonstrate the potential of chemotherapeutic agents such as cyclophosphamide and doxorubicin as inducers of WEE1 protein expression, potentially making them suitable for use in combination with AZD1775 in other cancers besides DLBCL.

Discussion

We presented highly robust data showing that the combination of the WEE1 inhibitor AZD1775 with RT or CHOP chemotherapy results in increased levels of DNA damage, activation of the DDR pathways and induction of premature mitotic entry, leading to synergistic lethality of DLBCL cells. Moreover, protein analysis revealed that RT, cyclophosphamide and doxorubicin all effectively increased WEE1 protein expression, indicating that these agents might be suitable for use in combination treatments with AZD1775.

The WEE1 tyrosine kinase plays an essential role in maintaining genomic stability by allowing DNA damage repair at the G2/M transition. Activation of WEE1 by CHK1 is a key mechanism preventing cell cycle progression during DNA damage repair.¹⁰ As DLBCL is a cancer with high levels of genomic instability¹³ and DNA damage,¹⁴ DLBCL cells rely heavily on checkpoint activation and DNA damage repair, which are effectively prevented by blocking WEE1. Furthermore, inhibition of CDC2/CDK1 by WEE1 during S-phase allows DNA replication and stabilization of replication forks, which become hazardous in the event of WEE1 inhibition.²⁰ In tumour cells lacking a mechanism for repair in G1/S phase as a result of TP53 mutation, cell cycle arrest and repair at the G2/M phase checkpoint is especially important²¹ These factors all contribute to making WEE1 an attractive target for inhibitor therapy in either TP53-deficient tumours or together with genotoxic therapy to either enhance DNA damage induction or block DNA damage repair. However, we found that TP53 mutation status did not determine sensitivity to the WEE1 inhibitor AZD1775 in our DLBCL cell lines, a finding that contradicts current literature in non-small cell lung cancer¹⁷ and ovarian adenocarcinoma tumour cells.¹⁵ However, other studies in acute myeloid leukemia (AML) have shown that TP53 does not

determine therapy outcome with AZD1775,^{22,23} indicating possible differences between solid tumours and hematologic malignancies.

High levels of synergism and DNA damage were observed across multiple cell lines, likely due to the high levels of genomic instability and DNA damage outweighing the presence of TP53 in DLBCL.

Chemotherapy in combination with AZD1775 has been shown to be synergistic together with compounds such as doxorubicin in colon cancer cell lines²⁴ and B-cell lymphoma cell lines,²⁵ with cytarabine in B-cell lymphoma cell lines and xenograft mouse models,²⁵ vincristine in B-cell and T-cell leukaemia cell lines and patient cells,²⁶ cyclophosphamide-like compounds in lymphoblastoid cell lines and rhabdomyosarcoma cell lines^{27,28} and with paclitaxel in breast cancer.²⁹ Similarly, combined WEE1 inhibition and RT has proven successful in gliomas and osteosarcoma.^{30,31} Recently, Wang and colleagues showed that the efficacy of doxorubicin with AZD1775 was dependent on the moment of cell cycle arrest induced by doxorubicin, with combination therapy proving highly effective in G2/M phase-arrested lymphoma cell lines but not in G1/S-phase-arrested cells.²⁶ At the same time, doxorubicin was also shown to influence WEE1 and pCDC2 levels based on TP53 status in a time-dependent manner in melanoma cell lines. In TP53 wild type cells, doxorubicin induced a steady increase over the first 1–9 h after treatment, but both WEE1 and pCDC2 levels declined at 24 h. By contrast, pCDC2 levels steadily increased in TP53 mutant cells after treatment, even at 24 h.³² A comparable synergy with AZD1775 was shown for ionizing radiation, which causes predominantly single stranded DNA breaks,³³ leading to ATR activation and downstream phosphorylation and activation of CHK1.^{34,35} Since CHK1 is one of the most important positive regulators of WEE1,¹⁰ any activation of the ATR-CHK1 axis will likely result in increased activation of WEE1 and will likely benefit from AZD1775 combination treatment. We found enhanced toxicity for combinations of AZD1775 with RT, CHOP and single agent doxorubicin, which all resulted in G2/M arrest, increased WEE1 protein levels and high levels of DNA damage. Based on current evidence, synergy with AZD1775 seems likely when a treatment induces either: (a) G2/M arrest, (b) WEE1

upregulation, (c) activation of the DDR, or (d) all of the above. An exception to this rule was vincristine combined with AZD1775, as vincristine led to decreased protein levels of WEE1 and pCDC2. Visconti and colleagues showed that at the spindle assembly checkpoint during mitosis, WEE1 promotes survival when cells are under pressure from anti-microtubule cancer drugs or malformed spindles, and that this checkpoint is only restored through genetic or chemical inhibition of WEE1. In line with their hypothesis, Visconti and colleagues found synergism for the combination of vincristine and AZD1775, but no downregulation of WEE1 protein was observed upon treatment with 25 nM vincristine.²⁷ Although vincristine reduced WEE1 protein levels in our DLBCL cell line, these effects are likely counteracted by induction of WEE1 by doxorubicin and cyclophosphamide, therefore maintaining high levels of toxicity and synergism with AZD1775 in the CHOP combination.

In conclusion, we demonstrated that AZD1775 combined with RT and CHOP effectively inhibits WEE1 and is synergistic in multiple cell lines. Based on protein, cell cycle and DNA damage analysis, we predict that AZD1775 will be a highly effective treatment in combination with other (chemo) therapeutic compounds that induce either WEE1 upregulation, G2/M arrest or activation of the DDR. These data underline the considerable potential of WEE1 inhibition in the clinical setting and help pave the way for the application of AZD1775 and chemotherapeutic combination therapies in other cancer types.

Acknowledgements

We thank the University Medical Center Groningen Pharmacy and flow cytometry department for support and technical help. We apologize to those whose work was not cited here due to length restrictions.

Author contributions

MRWdJ, BR, PH and ML performed the *in vitro* work and analysed the data. ML performed the TP53 mutation analysis. MRWdJ, TvM, LV, MN, GH, EA and AvdB designed the research, analysed the data and wrote the manuscript.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research

was supported by a Bas Mulder grant of Alpe d'HuZes/Dutch Cancer Society (RUG 2014-6727) and a Mandema Stipendium (awarded to T. van Meerten).

Conflict of interest statement

The authors declare that there is no conflict of interest.


Ethics approval and consent to participate


Not applicable.

Consent for publication

All authors have read, understood and approved the authorship agreement.

ORCID iDs

Mathilde Rikje Willemijn de Jong  <https://orcid.org/0000-0002-0427-6384>

Tom van Meerten  <https://orcid.org/0000-0002-5581-6950>

Availability of data and materials

Data, samples or materials will be made available upon request and can be acquired through contact with the corresponding author.

Supplemental material

Supplemental material for this article is available online.

References

- Jeong JU, Chung WK, Nam TK, *et al.* Treatment results of radiotherapy following CHOP or R-CHOP in limited-stage head-and-neck diffuse large B-cell lymphoma: a single institutional experience. *Radiat Oncol* 2017; 35: 317–324.
- Tilly H, Gomes da Silva M, Vitolo U, *et al.* Diffuse large B-cell lymphoma (DLBCL): ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2015; 26(Suppl. 5): v116–v125.
- Chiappella A, Martelli M, Angelucci E, *et al.* Rituximab-dense chemotherapy with or without high-dose chemotherapy plus autologous stem-cell transplantation in high-risk diffuse large B-cell lymphoma (DLCL04): final results of a multicentre, open-label, randomised, controlled, phase 3 study. *Lancet Oncol* 2017; 18: 1076–1088.
- van Imhoff GW, McMillan A, Matasar MJ, *et al.* Ofatumumab versus rituximab salvage chemoimmunotherapy in relapsed or refractory diffuse large b-cell lymphoma: the ORCHARRD study. *J Clin Oncol* 2017; 35: 544–551.
- Vitolo U, Trneny M, Belada D, *et al.* Obinutuzumab or rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone in previously untreated diffuse large B-cell lymphoma. *J Clin Oncol* 2017; 35: 3529–3537.
- Leonard JP, Kolibaba KS, Reeves JA, *et al.* Randomized phase II study of R-CHOP with or without bortezomib in previously untreated patients with non-germinal center B-cell-like diffuse large B-cell lymphoma. *J Clin Oncol* 2017; 35: 3538–3546.
- Nowakowski GS, LaPlant B, Macon WR, *et al.* Lenalidomide combined with R-CHOP overcomes negative prognostic impact of non-germinal center B-cell phenotype in newly diagnosed diffuse large B-Cell lymphoma: a phase II study. *J Clin Oncol* 2015; 33: 251–257.
- Younes A, Thieblemont C, Morschhauser F, *et al.* Combination of ibrutinib with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) for treatment-naive patients with CD20-positive B-cell non-Hodgkin lymphoma: a non-randomised, phase 1b study. *Lancet Oncol* 2014; 15: 1019–1026.
- de Jong MRW, Visser L, Huls G, *et al.* Identification of relevant drugable targets in diffuse large B-cell lymphoma using a genome-wide unbiased CD20 guilt-by association approach. *PLoS One* 2018; 13: e0193098.
- Lee J, Kumagai A and Dunphy WG. Positive regulation of WEE1 by Chk1 and 14-3-3 proteins. *Mol Biol Cell* 2001; 12: 551–563.
- Barr AR, Cooper S, Heldt FS, *et al.* DNA damage during S-phase mediates the proliferation-quiescence decision in the subsequent G1 via p21 expression. *Nat Commun* 2017; 8: 14728.
- Gu X, Booth CJ, Liu Z, *et al.* AID-associated DNA repair pathways regulate malignant transformation in a murine model of BCL6-driven diffuse large B-cell lymphoma. *Blood* 2016; 127: 102–112.
- Barlow JH, Faryabi RB, Callen E, *et al.* Identification of early replicating fragile sites that contribute to genome instability. *Cell* 2013; 152: 620–632.

14. Derenzini E, Agostinelli C, Imbrogno E, *et al.* Constitutive activation of the DNA damage response pathway as a novel therapeutic target in diffuse large B-cell lymphoma. *Oncotarget* 2015; 6: 6553–6569.
15. Hirai H, Iwasawa Y, Okada M, *et al.* Small-molecule inhibition of WEE1 kinase by MK-1775 selectively sensitizes p53-deficient tumour cells to DNA-damaging agents. *Mol Cancer Ther* 2009; 8: 2992–3000.
16. Habermann TM, Weller EA, Morrison VA, *et al.* Rituximab-CHOP versus CHOP alone or with maintenance rituximab in older patients with diffuse large B-cell lymphoma. *J Clin Oncol* 2006; 24: 3121–3127.
17. Do K, Wilsker D, Ji J, *et al.* Phase I study of single-agent AZD1775 (MK-1775), a WEE1 kinase inhibitor, in patients with refractory solid tumours. *J Clin Oncol* 2015; 33: 3409–3415.
18. Bartek J and Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* 2003; 3: 421–429.
19. Liston DR and Davis M. Clinically relevant concentrations of anticancer drugs: a guide for nonclinical studies. *Clin Cancer Res* 2017; 23: 3489–3498.
20. Beck H, Nahse-Kumpf V, Larsen MS, *et al.* Cyclin-dependent kinase suppression by WEE1 kinase protects the genome through control of replication initiation and nucleotide consumption. *Mol Cell Biol* 2012; 32: 4226–4236.
21. Senturk E and Manfredi JJ. p53 and cell cycle effects after DNA damage. *Methods Mol Biol* 2013; 962: 49–61.
22. 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: 2675–2684.
23. Garcia TB, Snedeker JC, Baturin D, *et al.* A small-molecule inhibitor of WEE1, AZD1775, synergizes with olaparib by impairing homologous recombination and enhancing DNA damage and apoptosis in acute leukemia. *Mol Cancer Ther* 2017; 16: 2058–2068.
24. Hirai H, Arai T, Okada M, *et al.* MK-1775, a small molecule WEE1 inhibitor, enhances anti-tumour efficacy of various DNA-damaging agents, including 5-fluorouracil. *Cancer Biol Ther* 2010; 9: 514–522.
25. Wang X, Chen Z, Mishra AK, *et al.* Chemotherapy-induced differential cell cycle arrest in B-cell lymphomas affects their sensitivity to WEE1 inhibition. *Haematologica* 2018; 103: 466–476.
26. Visconti R, Della Monica R, Palazzo L, *et al.* The Fcp1-WEE1-Cdk1 axis affects spindle assembly checkpoint robustness and sensitivity to antimicrotubule cancer drugs. *Cell Death Differ* 2015; 22: 1551–1560.
27. Goldstein M, Roos WP and Kaina B. Apoptotic death induced by the cyclophosphamide analogue mafosfamide in human lymphoblastoid cells: contribution of DNA replication, transcription inhibition and Chk/p53 signaling. *Toxicol Appl Pharmacol* 2008; 229: 20–32.
28. Kahen E, Yu D, Harrison DJ, *et al.* Identification of clinically achievable combination therapies in childhood rhabdomyosarcoma. *Cancer Chemother Pharmacol* 2016; 78: 313–323.
29. Lewis CW, Jin Z, Macdonald D, *et al.* Prolonged mitotic arrest induced by Wee1 inhibition sensitizes breast cancer cells to paclitaxel. *Oncotarget* 2017; 8: 73705–73722.
30. Caretti V, Hiddingh L, Lagerweij T, *et al.* WEE1 kinase inhibition enhances the radiation response of diffuse intrinsic pontine gliomas. *Mol Cancer Ther* 2013; 12: 141–150.
31. PosthumaDeBoer J, Wurdinger T, Graat HC, *et al.* WEE1 inhibition sensitizes osteosarcoma to radiotherapy. *BMC Cancer* 2011; 11: 156.
32. Vera J, Raatz Y, Wolkenhauer O, *et al.* Chk1 and WEE1 control genotoxic-stress induced G2-M arrest in melanoma cells. *Cell Signal* 2015; 27: 951–960.
33. Lankinen MH, Vilpo LM and Vilpo JA. UV- and gamma-irradiation-induced DNA single-strand breaks and their repair in human blood granulocytes and lymphocytes. *Mutat Res* 1996; 352: 31–38.
34. Shiotani B and Zou L. Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks. *Mol Cell* 2009; 33: 547–558.
35. Zhao H and Piwnicka-Worms H. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. *Mol Cell Biol* 2001; 21: 4129–4139.