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Targeting WEE1 in diffuse large B-cell lymphoma: mediator in DNA damage and apoptosis

de Jong, Mathilde

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Targeting WEE1 in diffuse large B-cell lymphoma: mediator in DNA damage and apoptosis

Mathilde Rikje Willemijn de Jong

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Introduction and scope of this thesis

Introduction

Diffuse large B-cell lymphoma

Diffuse large B-cell lymphoma (DLBCL) is the most common form of non-Hodgkin lymphoma and is histologically characterized by a diffuse proliferation of large neoplastic B cells with a nuclear size equal to or exceeding normal nuclei. The disease has an incidence of 7–8 cases per 100,000 people per year in the USA and the UK, and although the disease can arise at any age, DLBCL is mostly found in elderly patients with an average age of 70 years old [1]. Because of its aggressive nature, the first symptoms of DLBCL are often the discovery of a fast-growing mass combined with fever, weight loss and night sweats, which are classified as B-symptoms. DLBCL arises as the result of a malignant transformation of B-cells, or as the result of a transformation of other types of lymphoma or leukemia, such as follicular lymphoma (FL) or chronic lymphocytic leukemia (CLL). Based on pathological classification or site of presentation, DLBCL is divided in several subtypes which include amongst others primary DLBCL of the central nervous system (CNS), T-cell/histiocyte-rich large B-cell lymphoma and intravascular large B-cell lymphoma. The most common subtype is the DLBCL not otherwise specified (NOS) group, including cases that do not fit into any specific disease subgroup.

Genetic background and subclassification

Although DLBCL is a very heterogeneous disease, it can be subdivided into three different subcategories based on gene-expression profiles, which reflect the cell of origin (COO) of the maturation stage of the precursor B cell. These subtypes are classified as the germinal center B-cell (GCB) subtype, activated B-cell (ABC) or non-GCB subtype and the third unclassified subtype, which each have a unique gene-expression profile and differ in prognosis for survival [2]. The more common GCB subtype is known for its expression of cell surface enzyme CD10 and transcription factors LMO2 and BCL-6 [3,4]. Approximately 30% of GCB-DLBCLs have a (14;18) translocation, resulting in the translocation of the anti-apoptotic BCL-2 gene locus to the immunoglobulin heavy chain gene (IGH) enhancer locus, inducing BCL-2 overexpression and inhibition of apoptosis [5]. Similarly, the (8;14) translocation of the MYC gene locus to the IGH enhancer induces overexpression of the oncogene MYC resulting in an enhanced cellular proliferation rate. The MYC translocation can be found together with a BCL-2 translocation in about 10% of DLBCL cases, which are referred to as high grade B-cell lymphoma with rearrangement or “double-hit” cases [6]. Other characteristics of GCB-DLBCL include mutations of the histone methyltransferase EZH2 gene and loss of tumor suppressor gene PTEN, resulting in increased transcriptional repression of tumor suppressors and decreased inhibition of the Akt/PKB pro-survival signaling pathway, respectively [7]. The gene expression profile of the GCB subtype shows great overlap with normal germinal center B-cells, suggesting that the GCB subtype arises at an early stage in

B-cell development in the germinal center [8]. Besides the GCB subtype, gene expression profiling also recognizes the more aggressive non-GCB subtype or activated B-cell (ABC) subtype. These tumors arise from the activated plasmablast B-cell stage, which is the maturation stage of B-cells just prior to the germinal center exit [8]. Thus, non-GCB cells are in a more “activated” state and often show constitutive activation of the NF- κ B signaling pathway, promoting cell survival, proliferation, and inhibition of apoptosis [9]. This NF- κ B activation can be the result of mutations in the adaptor protein MYD88, CD79A or CD79B, or via activation of the CBM signaling complex (formed by CARD11, BCL10, and MALT1), constitutive active B-cell receptor (BCR) signaling, or activation of downstream kinases such as SYK, PI3K and BTK [10]. Because of this highly active state, non-GCB DLBCL patients often have a worse prognosis compared to GCB DLBCL patients, with 3-year overall survival rate of 56% versus 84% ($p > 0.001$) [2]. Last but not least, a small group of DLBCL patients remains unclassified, which are believed to represent lymphomas arising from different stages of B cell differentiation/maturation.

DNA damage and DLBCL

An important feature shared by both GCB and non-GCB DLBCL are the high levels of genomic instability, which results in constitutive high expression and activation of the DNA damage response (DDR) pathway in DLBCL compared to other cancers [11]. The DNA damage response pathway is multi layered pathway which can be subdivided into three basic steps of 1) detection of DNA damage by “sensor” proteins (e.g. RPA, γ H2AX or the MRN complex) and “mediator” proteins (e.g. BRCA1/2), which is followed by 2) activation of downstream “transducer” proteins (e.g. ATM or ATR), and leads to activation of 3) “effector” proteins that are involved in DNA repair, cell cycle regulation and apoptosis (Figure 1).

Depending on the type of DNA damage and/or the timing it occurred, DNA damage can be detected and repaired by several different players of the DNA damage response pathways. In general, acquisition of single stranded DNA damage allows for relative error-free repair, as the remaining DNA strand can serve as an accurate template. In this situation, several repair options are available, which include base excision repair (BER), nucleotide excision repair (NER) or mismatch repair (MMR). Initial steps in the detection and repair of single stranded breaks (SSB) and/or replication stress require recognition by replication protein A (RPA) and activation of kinases ataxia telangiectasia and rad3-related protein (ATR) and checkpoint kinase 1 (CHK1), forming the ATR-CHK1 axis [12]. The most common inducer of single stranded breaks is oxidative stress or reactive oxygen species (ROS). In addition, they can also be induced by X-ray radiation or alkylating agents [13].

In the situation of double stranded DNA damage, the mechanisms of repair are dependent on the presence or absence of a DNA template: if cells have completed DNA replication in S-phase, a template is available to allow the error-free homologous recombination (HR), which is similar to the chromosomal crossover during meiosis. In absence of a template, damage can be repaired using either non-homologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ). Initial steps in the repair of double

stranded breaks (DSB) require the recognition of double stranded breaks (DSB) by the kinase ataxia telangiectasia mutated (ATM) and the MRE11:RAD50:NBS1 (MRN) sensor complex [14], resulting in phosphorylation of DNA damage-associated γ H2AX histone mark and activation of checkpoint kinase 2 (CHK2), together forming the ATM-CHK2 pathway [12].

Figure 1. Simplified schematic overview of the multiple pathways activated by DNA damage. Induction of DNA damage result in activation of sensors and mediators of either double stranded (i.e. MRN complex, γ H2AX) or single stranded DNA breaks (i.e. RPA), which activate downstream transducers ATM and/or ATR. These transducers are responsible for the initiation of pathways for repair, cell cycle checkpoints and if necessary, apoptosis.

Double stranded DNA breaks (DSB) are mostly induced by ionizing radiation and chemotherapeutic drugs, such as DNA-alkylating agents (cyclophosphamide), DNA cross-linking agents (cisplatin) and radiomimetic compounds (bleomycin or phleomycin) (15). Together these different pathways are able to recognize and repair DNA damage and prevent abnormal cellular functioning.

Simultaneously with the initiation of the DNA damage repair pathway, the ATM and ATR transducers also activate pathways to regulate cell cycle progression (Figure 2). Activation of cell cycle checkpoint proteins is necessary to allow time for repair and prevent progression into mitosis and cell division, which would lead to permanently damaged DNA into the next generation of cells. The most important cell cycle regulator in this pathway is WEE1, which is part of the WEE family of kinases that function as regulators for cell cycle progression. Members of this family include WEE1 (WEE1A), MYT1 (PKMYT1) [16] and the oocyte-specific WEE2 (WEE1B) which regulates meiosis [17]. The main function of WEE1 concerns phosphorylation of cyclin dependent kinase 1 (CDK1/CDC2) on tyrosine 15, thereby inhibiting CDK1 activity and preventing regular transition from G2 phase into the M phase until cells are ready for mitosis [18]. At the same time, WEE1 has shown to control CDK2 activity during DNA replication in S-phase, thereby preventing excessive firing of replication origins, preventing DNA replication stress and promoting homologous recombination [19-

21]. Opposite of the WEE kinases are the phosphatases of the cell division cycle 25 (CDC25) family, which include CDC25A, CDC25B and CDC25C, and are responsible for the removal of the inhibitory phosphate residues from the CDK active site [22].

Figure 2. Overview of the cross-talk between the DNA damage response and the cell cycle regulator WEE1. Depicted are the ATM-CHK2 and ATR-CHK1 pathways which are involved in repair of double stranded (DS) and single stranded (SS) DNA breaks, respectively. Inactivation of the CDC25 family members and/or activation of WEE1 by CHK1 prevents G2/M progression upon DNA damage, couples the DNA damage response to cell cycle, thereby allowing DNA repair.

In the case of DNA damage inflicted during or at the end of the G2 phase, cells will activate the G2-checkpoint which checks for cell size and DNA integrity [23]. As a result, activation of the ATR-CHK1-pathway will result in activation of WEE1 by CHK1 in order to prevent unwanted cell cycle progression and allow time either repair the inflicted damage or induce apoptosis [24]. In addition, activation of ATM/ATR results in inactivation of CDC25C to prevent removal of the inhibitory phosphate added by WEE1 [25,26]. This makes WEE1 an essential link between cell cycle and DNA damage pathways, by preventing unscheduled cell cycle progression and allowing time for critical DNA damage repair. In this rather complex and extensive network of cell cycle and DNA damage related proteins, many factors have shown to be either mutated or aberrantly expressed in DLBCL (Figure 3). Clinical analysis of DDR proteins in DLBCL through immunohistochemistry found positive staining for γH2AX, CHK1/2 and CDC25C in half of the DLBCL cases, which was significantly higher compared to the percentages of positive cases observed in indolent B-cell lymphomas such as marginal zone lymphoma (MZL) and chronic lymphocytic leukemia (CLL) [11]. Moreover, mutation studies in DLBCL samples revealed recurrent mutations in DNA damage repair genes, including mismatch repair genes (EXO1, MSH2, and MSH6) and NHEJ genes (DCLRE1C/ARTEMIS, PRKDC/DNA-PKcs, XRCC5/KU80 and XRCC6/KU70) [27,28]. In addition, Bret et al. showed that the expression pattern of 176 genes involved in FANC, NER, HR, BER, NHEJ and MMR pathways was associated with survival in DLBCL patients [29].

Figure 3. Schematic representation of the affected DNA damage response proteins in DLBCL. Depicted are the sensors, mediators, transducers and effectors involved in the DNA damage response, which show aberrant gene expression, protein expression or high incidence of mutations in DLBCL. Depicted in red are the cell cycle checkpoints that determine whether cells can progress into the next phase of the cell cycle.

DNA damage and the germinal center reaction

As previously mentioned, DLBCL show a significantly higher incidence of genetic instability as compared to many other cancers (Figure 2). As a result of this genetic instability, DLBCL are characterized by high percentages of translocations and mutations, which leads to the activation of DNA damage pathways. The genomic instability is the result of high activity of activation-induced cytidine deaminase (AID). AID is an enzyme required for somatic hypermutation (SHM) and class-switch recombination (CSR) which facilitate the rearrangement of the immunoglobulin genes to generate antigen-specific, high-affinity, class-switched B-cells [30]. However, aberrant and off-target activity of AID and other enzymes involved in these processes, are major drivers of DLBCL [31]. Immunohistochemical staining of AID in DLBCL samples demonstrated high expression in ~40% of DLBCL patients and its expression was associated with poor survival [32]. In addition, it was shown that AID gene expression was detected in both GCB- and non-GCB DLBCL and was decreased in DLBCL that evolved from follicular lymphoma (FL) [33]. Moreover, AID expression was not associated with the degree of intra-clonal heterogeneity. In mice experiments, AID deficiency prevented formation of BCL-6-dependent GC B-cell derived NHL, but it had no impact on MYC-driven

pre-GC lymphomas [34]. In contrast, Compagno et al. showed a direct link between AID activity and genomic instability, in which activation of AID through PI3 kinase blockade resulted in increased genome-wide off-target chromosomal translocations and increased incidence of plasma cell tumors in mice [35]. Taken together, these data demonstrate a pivotal role of AID in the transformation of normal GC B-cells into a tumorigenic state, in which AID remains to be activated in cells which may otherwise have lost expression of AID and other germinal center-related genes.

Treatment

Despite an increase in the knowledge for DLBCL genetic subtypes, activated pathways and differential clinical features, treatment of DLBCL has remained unchanged in the last decade. For a long period of time, treatment for DLBCL has consisted of the chemotherapeutic combination of cyclophosphamide, doxorubicin, vincristine and prednisolone to create CHOP. With the discovery of the favorable results of the anti-CD20 antibody rituximab on survival of lymphoma patients, the combined R-CHOP treatment was approved and has been used as standard treatment for DLBCL since 1997 [36]. CD20 is an activated-glycosylated phosphoprotein expressed at the membrane of all B-cells, and is thought to play a role in B-cell activation, differentiation, and cell-cycle progression [37]. Addition of anti-CD20 antibody rituximab to the CHOP regimen has increased the overall 5-year survival from 40% to 60% in DLBCL patients [38]. Despite this improvement, a large portion of patients still respond poorly, with ~20% of DLBCL patients having refractory disease and 30% with relapse disease. For these patients, limited treatment options are available, which include salvage chemotherapy or autologous stem cell transplantation [39]. Overall, treatment for DLBCL remains inadequate and burdensome, and therefore novel therapies are required, preferentially therapies that focus on the specific biology of DLBCL or focus on specific therapy-induced intra-cellular changes.

Scope of this thesis

Based on the current response rate of DLBCL patients to standard R-CHOP treatment it is clear that novel targets are needed to improve therapy outcome for these patients. With the knowledge that DNA damage response and cell cycle pathways play important roles in either the initiation or progression of DLBCL, it is attractive to explore feasibility of targeting these pathways. We therefore aimed to explore the DNA damage response pathway, in both its intrinsic biology and as a target for therapy. In order to answer these questions, we first investigated whether DNA damage response and cell cycle proteins were associated with CD20 expression in DLBCL, and whether these could be efficiently targeted for therapy (Chapter 2). This association with CD20 was chosen because CD20 is not only an important

target for therapy with the mono-clonal antibody rituximab, but also because CD20 is closely involved in signaling through the B-cell receptor (BCR), which is often a driver pathway in DLBCL. Based on these findings, we selected cell cycle regulator WEE1 to further study in depth as a target for combination therapy. Since our aim is to improve therapy response and reduce treatment burden, we investigated the potential of WEE1 inhibition in combination with first line radiation and chemotherapy (Chapter 3) and in combination with second line cytarabine and cisplatin treatment (Chapter 4). In addition, we tested whether WEE1 inhibition would synergize with novel therapeutic compounds that target the cell cycle through inhibition of cyclin-dependent kinases (CDKs), which are currently tested in clinical trials (Chapter 5).

Finally, in order to define the role of the anti-apoptotic pathways that allow tumor survival and resistance in DLBCL (such as BCL-2), we explored the effects of WEE1 inhibition (Chapter 6) and CHOP chemotherapy (Chapter 7) on the anti-apoptotic dependency, allowing us to determine the potential efficacy of anti-apoptotic inhibitors in DLBCL treatment. In the final Chapter 8, we summarize and discuss our findings and provide future perspectives for WEE1 in treatment of patients with DLBCL and other cancers.

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Identification of relevant drugable targets in diffuse large B-cell lymphoma using a genome-wide unbiased CD20 guilt- by-association approach

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Abstract

Forty percent of patients with diffuse large B-cell lymphoma (DLBCL) show resistant disease to standard chemotherapy (CHOP) in combination with the anti-CD20 monoclonal antibody rituximab (R). Although many new anti-cancer drugs were developed in the last years, it is unclear which of these drugs can be safely combined to improve standard therapy without antagonizing anti-CD20 efficacy. In this study, we aimed to identify rituximab compatible drug-target combinations for DLBCL. For this, we collected gene expression profiles of 1,804 DLBCL patient samples. Subsequently, we performed a guilt-by-association analysis with MS4A1 (CD20) and prioritized the 500 top-ranked CD20-associated gene probes for drug-target interactions. This analysis showed the well-known genes involved in DLBCL pathobiology, but also revealed several genes that are relatively unknown in DLBCL, such as WEE1 and PARP1. To demonstrate potential clinical relevance of these targets, we confirmed high protein expression of WEE1 and PARP1 in patient samples. Using clinically approved WEE1 and PARP1 inhibiting drugs in combination with rituximab, we demonstrated significantly improved DLBCL cell killing, also in rituximab-insensitive cell lines. In conclusion, as exemplified by WEE1 and PARP1, our CD20-based genome-wide analysis can be used as an approach to identify biological relevant drug-targets that are rituximab compatible and may be implemented in phase 1/2 clinical trials to improve DLBCL treatment.

Introduction

Diffuse Large B-cell lymphoma (DLBCL) is the most common type of Non-Hodgkin lymphoma (NHL). Standard immunochemotherapy consisting of cyclophosphamide, doxorubicin, vincristine, and prednisolone combined with the anti-CD20 monoclonal antibody rituximab (R-CHOP) results in a cure rate of 60% [1]. However, 40% of patients have refractory or relapsing disease and their prognosis is poor [2]. Unfortunately, since the introduction of rituximab two decades ago, all efforts to intensify chemotherapy or develop next generations anti-CD20 antibodies failed to improve their survival [3–5]. For these patients, there is an unmet need to improve standard treatment for DLBCL.

The B-cell receptor (BCR) complex, with the CD20 protein—a product of the *MS4A1* gene—as a part of the BCR signalosome [6], is recognized as an important pathway that drives tumor growth and survival of various B-cell NHLs [7,8]. It has been demonstrated that DLBCL shows the highest basal phosphorylation levels of the BCR complex compared to other B-cell malignancies [9], and that the ongoing antigenic engagement of self-antigens on the BCR is required for tumor survival in activated B-cell (ABC) subtype DLBCL [10]. Emerging data from clinical trials indicate that blocking kinases downstream of the BCR has substantial anti-lymphoma activity. For example, inhibition of BTK, PI3K and SYK through ibrutinib [11,12], idelalisib [13], and fostamatinib [14,15], respectively, has been shown to be effective in follicular lymphoma, mantle cell lymphoma (MCL), and chronic lymphocytic leukemia (CLL). The efficacy of rituximab depends on CD20 clustering within the BCR, whereby rituximab also activates complement in a BCR-dependent manner [16]. In addition, CD20 ligation with monoclonal antibodies on NHL cell lines downregulates important components of the BCR signaling pathway [17,18]. Indeed, kinase inhibitors downstream of the BCR have been shown to interfere with the activity of rituximab [19–22]. Therefore, it is preferred to identify new drug targets for DLBCL outside the context of the CD20/BCR-signalosome.

In the present study, we aimed to identify therapeutic targets for combination therapy in DLBCL, which would be likely to improve treatment outcome without antagonizing the efficacy of rituximab. We therefore collected a large compendium of DLBCL gene expression profiles (GEPs) from the public domain and performed a guilt-by-association analysis with *MS4A1*. Subsequently, after the identification of the well-known but also several unknown DLBCL genes in association with CD20, we prioritized the top-ranked genes for drug-target interaction. Then, as an example, we confirmed high protein expression of two new target genes, *WEE1* and *PARP1*, in DLBCL patient samples. As a next step we combined clinically available inhibiting drugs for these targets with rituximab, which resulted in improved DLBCL cell killing.

Materials and methods

Data acquisition and sample processing and quality control

Publicly available raw microarray expression data of DLBCL samples were extracted from the Gene Expression Omnibus (GEO) [23]. The analysis was confined to the Affymetrix HG-U133A (GPL96) and Affymetrix HG-U133 Plus 2.0 (GPL570) platforms. Non-corrupted raw data CEL files were downloaded from GEO for the selected samples. To identify samples that have been uploaded to GEO multiple times we generated a MD5 (message-digest algorithm 5) hash for each individual CEL file. Before these MD5 hashes were generated we converted all CEL files to the GCOS XDA binary file format (version 4), which was done using the Affymetrix Power Tools (version 1.15.2) apt-cel-convert tool. A MD5 hash acts like a unique fingerprint for each individual file and duplicate CEL files will have an identical MD5 hash. After removal of duplicate CEL files, pre-processing and aggregation of CEL files was performed with RMAExpress (version 1.1.0) by applying the robust multi-array average (RMA) algorithm, using the latest Affymetrix GeneChip Array CDF layout files REF. Principal Component Analysis (PCA) on the sample correlation matrix was used for quality control. The first principal component (PCqc) of such an expression microarray correlation matrix nearly always describes a constant pattern that dominates the data, explaining around 80–90% of the total variance, which is independent of the biological nature of the sample being profiled. The correlation of each microarray expression profile with this PCqc can be used to detect outliers, as arrays of lesser quality will have a lower correlation with the PCqc. We removed samples that had a correlation $R < 0.8$. To minimize false positive or negative associations due to batch effects (different platforms and experiments) we calculated association statistics within meta-analysis batches. The combination of platform identifier (GPL number, i.e. GEO platform accession number) and experiment identifier (GSE number, i.e. GEO experiment accession number) were defined a meta-analysis batch. Meta-analysis statistic and p -values were calculated according to the generic inverse method with fixed effect model. To assess the degree of multiple testing, we performed this meta-analysis within a multivariate permutation test with 1000 permutation, a false discovery rate of 1% and a confidence level of 99%. For a detailed description we refer to our previous publication [24].

CD20 (MS4A1) guilt-by-association analysis

Probes representing MS4A1 were collapsed according to the mean. Next, we used mRNA signals to determine the association of each gene with the expression pattern of MS4A1. The association was determined by the Pearson correlation coefficient. Gene set enrichment analyses (GSEA) were performed on the 500 top-ranked MS4A1-associated probes (390 unique genes). The 390 MS4A1 co-expressed genes were uploaded to Enrichr [25], and several gene set databases were consulted (KEGG, Wiki pathways, Biocarta, NCI Nature,

Panther and GO biological process). To annotate a single gene to only one biological pathway, we manually marked single genes to 9 different biological pathways (BCR signaling, cytoskeleton regulation, DNA repair and cell cycle, histone modification, immune regulation, metabolism, protein processing, RNA processing, signaling protein (not further specified)).

Target prioritization

The 390 MS4A1-associated genes were analyzed in the drug-gene interaction database (DGidb) [26]. Next, by means of manual curation utilizing Pubmed, clinicaltrials.gov, and the websites of the American Society of Hematology, European Hematology Association, American Society of Clinical Oncology, and the European Society of Medical Oncology, we excluded the identified genes for which anti-neoplastic drugs had been previously investigated in clinical trials with DLBCL patients or already approved for clinical use in DLBCL.

Cell lines and culture conditions

DLBCL cell lines OCI-ly3, U-2932, SUDHL4 and SC-1 (all obtained from Deutsche Sammlung from Microorganism und Zellculturen, Braunschweig, Germany), SUDHL2 (obtained from American Type Culture collection, Manassus, Virginia, US) and Epstein-Barr virus transformed lymphoblastoid cells (LCL (LCL-1, LCL-2), immortalized from healthy volunteers, anonymized, obtained from A. van den Berg, University Medical Center Groningen [27]) were cultured in RPMI1640 (Lonza BioWhittaker, Walkersville, MD, USA) with 10% Fetal Bovine Serum (FBS; HyClone Thermo Scientific, Waltham, MA, USA), and DLBCL cell lines SUDHL5, SUDHL6 and SUDHL10 in RPMI1640 with 20% FBS. All cell lines were cultured at 37°C with 5% CO₂ in a humidified atmosphere and in 1% Penicillin-Streptomycin (Lonza Bio-Whittaker) and 1% Glutamine (Lonza BioWhittaker). The identity of our cell lines was checked periodically by STR profiling.

Western blot, patient material and immunohistochemistry

Cells were washed with PBS and lysed in RIPA buffer (50mM Tris/ 150mM NaCl/ 2.5mM Na₂EDTA/ 1% Triton X-100, 0.5% sodium deoxycholate/0.1% SDS in dH₂O) with 1mM phenylmethanesulphonyl fluoride for 30–45 minutes on ice. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (#23227; Thermo Scientific, Waltham MA, USA). Samples were loaded at 40µg 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 Milipore, Temecula, CA,

USA) and PARP1 (1:1000, #9542, Cell Signaling Technology, Danvers, MA, USA) was done overnight and staining for GAPDH (1:20,000; sc-47724 (0411), Santa Cruz Biotechnology, Dallas TX, USA) was done for one 1 hour at 4°C.

Randomly selected primary formalin fixed paraffin (FFPE) tissue from our anonymous tissue repository (Pathology, University Medical Center Groningen) was used of 16 primary DLBCL cases. The study protocol was consistent with international ethical and professional guidelines (the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice). The use of anonymous rest material is regulated under the code for good clinical practice in the Netherlands. Informed consent was waived in accordance with Dutch regulations.

Immunohistochemistry (IHC) was performed on FFPE tissue according to standard protocols with appropriate positive and negative controls (based on manufacturer's instructions). FFPE tissue of 16 randomly selected DLBCL patients was used. We used the following antibodies: anti-WEE1 (1:200, antigen retrieval with 10mM TRIS/ 1mM EDTA pH9 for 15 min at 120°C, one hour incubation at room temperature, Santa Cruz Biotechnology, Dallas TX, USA) and anti-PARP-1 (1:1000, antigen retrieval with 0.1M TRIS-HCL pH9 for 15 min at 120°C, incubation O/N at 4°C, Biorbyt, Cambridge, UK).

CD20 flowcytometry

A total of 0.1×10^6 cells were incubated with anti-CD20 (Clone B-Ly1 (R7013), Dako, Glostrup Municipality, Denmark) for 30 minutes on ice in the dark. After washing with 1% BSA in PBS cells were resuspended in 2% paraformaldehyde (Sigma) and analyzed for CD20 expression (mean fluorescence intensity (MFI)) with flow cytometry. To study the effect of PARP1 and WEE1 inhibition on CD20 expression levels, we determined CD20 expression levels with flow cytometry after AZD1775 (WEE1 inhibition) and olaparib (PARP1 inhibition) treatment after 48 hours. For WEE1 inhibition, 0.2 μ M AZD1775 for SUDHL6, SUDHL10 and SC-1 was used, and 1 μ M AZD1775 for U2932. For olaparib 20 μ M was used for SUDHL6, 50 μ M for SUDHL10 and SC-1, and 100 μ M for U9232.

Flow cytometry-based cytotoxicity assays

A total of 0.1×10^6 cells were pre-incubated with the inhibitor AZD1775 (WEE1 inhibitor, Selleckchem, Houston, TX, USA) for 48 hours at 37°C. After this pre-incubation 0 or 10 μ g/mL rituximab with 5% plasma (pooled plasma from 5 donors; Sanquin, the Netherlands) was added for 1 hour at 37°C. Next, cells were washed with 1% BSA in PBS and propidium iodide (Sigma, St. Louis MO, United States) was added for assessment of cell viability via flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes NJ, United States). Data were analyzed with Winlist 3D (Verity Software house, Topsham ME, USA). Cell lines were determined rituximab-sensitive when > 90% still have propidium iodide uptake upon rituximab treatment.

AZD1775 and olaparib dose optimization

The optimal concentration window for AZD1775 and olaparib was determined in rituximab sensitive and insensitive cell lines with flowcytometry assays as described above. AZD1775 was titrated in a range from 0.001 μM to 10 μM and olaparib in a range from 1 μM to 10.000 μM .

Statistical methods

All statistical analysis with respect to survival analysis and *in vitro* assays were undertaken using Graphpad PRISM software as detailed in Supplementary Methods. *P*-values <0.05 were considered significant.

Results

Data acquisition

Gene expression profiles of 1,804 DLBCL patients were collected from 20 studies (Supplemental table 1). For all patients metadata were also included (Figure 1). The majority of the DLBCL expression profiles originated from biopsies of lymph nodes (99%). For 93% of the cases a GEP-based cell-of-origin (COO) was provided, with 35% of the patients being classified as ABC DLBCL, 49% as Germinal Center B-cell (GCB) DLBCL, and 15% as unclassified DLBCL. Treatment data were available for 52% of the patients of which the majority (67%) received R-CHOP, and 33% received CHOP or an Acute Lymphoblastic Leukemia-like regimen. DLBCL patient characteristics are shown in Table 1.

MS4A1 guilt-by-association

To identify genes with similar expression patterns as MS4A1 we performed a guilt-by-association analysis. We identified 5,355 probes representing 3,893 unique genes that were significantly associated with MS4A1 (FDR 1%, CI 99%) (Figure 2A and Supplemental table 2). As expected, expression of several genes involved in BCR signaling such as CD79a, CD79b and CD22 was highly associated with MS4A1. For several of these genes, clinically-approved drugs are available and used to treat other types of cancer (Figure 2A). Fig 2A also shows targets that are under clinical evaluation for DLBCL, but for which expression is not associated with MS4A1, such as PIK3CA, BCL-2 or AKT1. Gene set enrichment analyses (GSEA) of the 500 top-ranked MS4A1-associated probes—representing 390 protein-coding

Figure 1. Work flow of the study. (A+B) 1804 Gene expression profiles (GEP) of patients with Diffuse Large B-cell Lymphoma from 20 studies were collected from the gene expression omnibus (GEO). (C) CD20 (gene: MS4A1), as a central protein in B-cell receptor (BCR) signaling and key target for the treatment of DLBCL, was chosen to perform a guilt-by-association analysis. Genes outside the context of BCR signaling (indicated by the grey dots) were chosen for drug-gene prioritization. (D) The Drug Gene Interaction database (DGIdb), Pubmed and clinicaltrials.gov were used to identify drug-gene targets that were not clinically studied in DLBCL before. (E) Two drug-gene targets were chosen for proof-of-concept in vitro studies.

genes—demonstrated a significant overrepresentation of the BCR signaling pathway according to multiple GSEAs with different gene set databases (e.g. KEGG $p=7.7 \times 10^9$, Wiki pathways $p=1.8 \times 10^{18}$, Biocarta $p=1.7 \times 10^6$, Supplemental table 2A–2F). To summarize the results of the GSEAs with different gene set databases, we annotated the 390 MS4A1 co-expressed gene set to 9 different biological pathways. Besides the well-known BCR signaling genes and immune regulation genes, other pathways included DNA repair and cell cycle, cytoskeleton regulation, metabolism and histone modification (Supplemental table 4). Correlation of the individual MS4A1-associated genes categorized by biological pathway is shown in Figure 2B. These 390 MS4A1 co-expressed genes include multiple potential targets for DLBCL treatment.

Target prioritization of MS4A1-associated genes

Next, the 390 MS4A1-associated gene set was prioritized for drug-gene interactions, to identify targets for which clinically-grade drugs are already available. At least 50 genes had one reported drug-target interaction (Supplemental table 5). Various genes belonging to the BCR signaling pathway were identified, such as like BTK, CD19, LYN, and SYK, which can be targeted with ibrutinib, SAR3419, ponatinib, and fostamatinib, respectively.

Table 1. Patient characteristics of the 20 collected DLBCL studies.

Clinical data	Number (and %) of available data	Characteristics of available clinical data
Age (years)	981 (54.5%)	
range		2–94 year
median		57.5 years
Sex	988 (54.8%)	
Male		437 (44.2%)
Female		551 (55.8%)
Ann Arbor	670 (37.1%)	
I		140 (20.9%)
II		175 (26.2%)
III		161 (24.0%)
IV		192 (28.7%)
IPI	570 (31.6%)	
0		66 (11.8%)
1		163 (28.6%)
2		160 (27.1%)
3		110 (19.3%)
4		60 (10.5%)
5		11 (1.9%)
Tissue	1796 (99.5%)	
Lymph node		1788 (99.5%)
Other		8 (0.4%)
Treatment	1113 (61.5%)	
CHOP		259 (23.3%)
R-CHOP		799 (71.9%)
Other		54 (4.9%)
Additional radiotherapy	158 (8.8%)	37 (23.4%)
Outcome	1016 (56.3%)	

Table 1 continued.

Clinical data	Number (and %) of available data	Characteristics of available clinical data
Cell-of-origin	1682 (93.2%)	
Activated B-cell		592 (35.2%)
Germinal Center B-cell		830 (49.3%)
Unclassified		260 (15.5%)
MYC rearrangement	283 (15.7%)	
MYC-neg		157 (55.5%)
IG-MYC		103 (36.4%)
Non-IG MYC		23 (8.1%)
Non-IG translocation BCL-2	286 (15.9%)	
BCL-2		44 (15.4%)
Non-IG BCL-2 expression	245 (13.6%)	
BCL-2 (pos)		166 (67.8%)
Non-IG translocation BCL-6	283 (15.7%)	
BCL-6		41 (14.5%)
Non-IG BCL-6 expression	231 (12.8%)	
BCL-6 (pos)		191 (82.7%)

In addition, we identified targets that interact with anti-neoplastic drugs that are currently used in treatment of DLBCL (e.g. DHFR interaction with methotrexate). We also observed targets that are involved in cellular energy metabolism interacting with non-cancer drugs (e.g. PRKAB1 with metformin, and PPP1CA with vitamin E). In addition, HDAC1 (panobinostat, belinostat, vorinostat, romidepsin), PSMD3 and PSMD6 (both carfilzomib) were identified as potential drugs for DLBCL treatment. These drugs are currently under clinical investigation in DLBCL. In Table 2, we summarize the identified drug-target combinations that, to our knowledge, have not been clinically studied in DLBCL patients, and do not interfere with the BCR signalosome. These drugs could potentially be introduced in clinical studies to improve DLBCL patient survival. The potential targets include DNA repair genes and cell cycle, such as PARP1, WEE1, CDK1, which can be targeted by olaparib, AZD1775 and dinaciclib respectively. Other genes are ESR2, (targeted by tamoxifen), PRKD3 (targeted by momelotinib), and BIRC3 (targeted by AT406). As proof-of-concept of our drug-discovery strategy, we selected WEE1 and PARP1, involved in cell cycle and DNA repair for further preclinical investigations.

Figure 2. MS4A1 guilt-by-association analysis. (A) Pearson's correlation plot of MS4A1 Guilt-by-Association of gene expression profiles of 1,804 DLBCL patient samples. In green, genes significantly positively associated with MS4A1, and in red, genes negatively associated with MS4A1. Several known and unknown genes in DLBCL are annotated in white (MS4A1-associated genes) and clear circles (drugable targets involved in clinical trials for diffuse large B-cell Lymphoma, but not highly associated with MS4A1). (B) The 500 top-ranked MS4A1 probes (representing 390 genes) were classified into 9 biological subgroups. This plot depicts genes within the subgroups associated to MS4A1 (Pearson correlation). The big dots represent genes for which clinical inhibitors are available.

Table 2. Drug-gene target prioritization.

<i>Gene</i>	<i>Location</i>	<i>Protein</i>	<i>Protein Function</i>	<i>Inhibitor</i>	<i>Clinical Use Inhibitor</i>
BIRC3	11q22	baculoviral IAP repeat containing 3	inhibits apoptosis by binding to tumor necrosis factor receptor-associated factors	AT-406	Ovarium cancer/ Acute myeloid Leukemia
PARP1	1q41-q42	poly (ADP-ribose) polymerase 1	repair of single-stranded DNA breaks	olaparib	Mammæ and prostate cancer
PRKD3	2p21	protein kinase D3	Binding of diacylglycerol and phorbol esters	momelotinib	Myelofibrosis
RP56 / IMPG2	3q12.2-q12.3	interphotoreceptor matrix proteoglycan 2	organization of the interphoto-receptor matrix and may promote the growth	PX-866	Non-small-cell lung cancer
WEE1	11p15.4	WEE1 G2 checkpoint kinase	tyrosine kinase, catalyzes the inhibitory tyrosine phosphorylation of CDC2/cyclin B kinase	AZD1775 / MK1775	Solid tumors
ESR2	14q23.3	estrogen receptor 2 (ER beta)	protein forms homo- or hetero-dimers that interact with specific DNA sequences to activate	tamoxifen	mammacarcinoma
CKD1	10q21.2	Cyclin-dependent kinase 1	Ser/Thr protein kinase family and catalytic subunit protein kinase complex known as M-phase promoting factor	Dinaciclib	Chronic Lymfocytic Leukemia and multiple myeloma
PDK3	Xp22.11	pyruvate dehydrogenase kinase, isozyme 3	nuclear-encoded mitochondrial multienzyme complex that catalyzes the overall conversion of pyruvate to acetyl-CoA and CO ₂	CPI-613	advanced hematologic malignancies
MAP3K1	5q11.2	mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase	serine/threonine kinase and is part of transduction cascades, including the ERK and JNK kinase pathways as well as the NF-kappa-B pathway	AZD8330	advanced malignancies

Relevance of WEE1 and PARP1 mRNA expression in DLBCL treatment

For both WEE1 and PARP1, mRNA expression was significantly higher within the GCB DLBCL subtype compared to ABC and unclassified subtypes (Kruskal-Wallis $p < 0.001$, Figure 3A and 3B). Survival and treatment data were available for 872 patients (R-CHOP and CHOP). Improved overall survival was observed in patients treated with R-CHOP compared to CHOP in DLBCL patients in all COO subgroups (Supplemental figure 1). The addition of rituximab to CHOP was markedly more beneficial in GCB-DLBCL patients with high WEE1 expression than in patients with low WEE1 expression (Hazard Ratio (HR) of 2.8, CI 1.5–5.1, $p = 0.001$ vs HR 2.0, CI 1.0–3.8, $p = 0.016$) (Figure 3C). For ABC-DLBCL patients with low or high WEE1 expression we observed no differences in the addition of rituximab to CHOP chemotherapy, respectively (HR of 2.2, CI 1.3–3.6, $p = 0.0008$ vs HR 2.0, CI 1.2–3.3, $p = 0.001$) (Figure 3D).

Figure 3. Expression levels of WEE1 and PARP1 in different DLBCL subgroups and in relation to anti-CD20 therapy with or without standard chemotherapy. (A) WEE1 and (B) PARP1 mRNA expression levels in Germinal Center B-cell (GCB, black), Activated B-cell (ABC, dark grey), and unclassified (light grey) Diffuse Large B- cell Lymphoma (DLBCL) samples. Overall survival for patients with DLBCL-GCB (C) and DLBCL-ABC (D) with low and high WEE1 expression treated with CHOP or R-CHOP, and overall survival for DLBCL-GCB (E) and DLBCL-ABC (F) patients with low and high PARP1 expression treated with CHOP or R-CHOP. Shown in the tables provided are the hazard ratios of adding anti-CD20 therapy with rituximab to standard chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP)). Log-rank testing was used to test whether the curves are statistically different and to calculate the hazard ratio's.

In GCB-DLBCL there were no differences in survival HRs for the addition of rituximab to CHOP in patients with high or low PARP1 expression (high PARP1: HR 2.3, CI 1.4–4.8, $p=0.003$ vs low PARP1 HR 2.6, CI 1.4–4.8, $p=0.0005$, Figure 3E). However, addition of rituximab to CHOP was markedly more beneficial with respect to survival in ABC-DLBCL patients with high PARP1 expression than in patients with low PARP expression (HR 2.8, CI 1.6–4.7. $p=0.001$ vs HR 1.6 CI 0.9–2.5 $p=0.04$) (Figure 3F). These data show that the additional effect of rituximab to CHOP may also be associated with the expression level of WEE1 and PARP1.

WEE1 and PARP1 protein expression and targeting of WEE1 and PARP1 kills DLBCL cell lines

Immunoblotting revealed WEE1 and PARP1 expression in all eight DLBCL cell lines, and not in control LCL cells (Figure 4A). In FFPE tissue samples both WEE1 and PARP1 showed a

nuclear staining pattern in tumor cells. WEE1 was expressed in 14 out of 16 cases (78%) and PARP1 in 15 out of 16 cases (94%), for both WEE1 and PARP1 the percentage of positive cells and protein intensity levels differed between patient samples (Figure 4B). This indicates that WEE1 and PARP1 are expressed at the protein level in DLBCL, both in DLBCL cell lines and primary cases.

Figure 4. Protein expression of WEE1 and PARP1 in DLBCL and in vitro killing assays. (A) Western blot results for Wee1, PARP1 in eight DLBCL cell lines. Two LCL cell lines are shown as normal B-cell controls. (B) Immunohistochemistry of Wee1 (left column) and PARP1 (right column) on DLBCL patient samples. Both Wee1 and PARP1 showed a nuclear staining pattern. (C) Cytotoxicity assays of the WEE1 inhibitor AZD1775 with or without rituximab in two rituximab sensitive and two resistant cell lines: SUDHL6 (rituximab sensitive, RS), SUDHL10 (RS), U2932 (rituximab insensitive, RI) and SC-1 (RI). Shown is the normalized live population (propidium iodide negative population) of three independent experiments. Student T-test was used to demonstrate significance (*) $p<0.05$ / (**) $p<0.005$. (D) Resazurin metabolic activity assay with the PARP1 inhibitor olaparib with or without rituximab in the above-mentioned cell lines. Shown is the normalized metabolic activity of three independent analyses. Student T-test was used to compare samples without inhibitor treatment. Significant (*) $p<0.05$ / (**) $p<0.005$ / (***) $p<0.001$.

Next, we tested the effect of WEE1 and PARP1 inhibitors on DLBCL cell lines as single agent and in combination with rituximab. Single agent rituximab killing assays demonstrated that 4 of the 8 DLBCL cell lines were sensitive to rituximab treatment, corresponding to CD20 expression levels (Supplemental figure 2A and Supplemental figure 2B). We selected 2 rituximab-sensitive (RS) (SUDHL6 and SUDHL10) and 2 rituximab-insensitive (RI) cell

lines (U2932 and SC-1) for further preclinical investigation. As a single agent, increasing concentrations of the WEE1 inhibitor AZD1775 strongly reduced cell viability in RS and RI cell lines after 48 hours (Figure 4C), without influencing CD20 expression levels (Supplemental figure 2C). Combining AZD1775 with rituximab showed a significant additional decline of cell survival in all tested cell lines (Figure 4C). In the DLBCL cell line SUDHL6 (RS), adding rituximab to a concentration of 2 μ M AZD1775 decreased cell viability from 18% to 5% ($p=0.0311$) compared to AZD1775 alone, for SUDHL10 (RS), adding rituximab to a concentration of 0.2 μ M AZD1775 decreased cell viability from 105% to 18% ($p=0.0015$) compared to AZD1775 alone, for the U2932 cell line (RI), cell viability decreased from 62% to 37% at 5 μ M ($p=0.00154$) compared to AZD1775 alone, and for SC-1 (RI), a concentration of 2 μ M AZD1775 plus rituximab decreased cell viability from 36% to 18% ($p=0.0039$). Similar results were obtained when WEE1 inhibition with rituximab was tested in the resazurin metabolic activity assay (Supplemental figure 2D). PARP1 inhibition by clinically obtained olaparib dose levels had limited single agent activity (Figure 4C). However, in cell viability assays combining 10 μ M olaparib with rituximab in SUDHL6 (RS) resulted in an additional decline in cell viability (75% to 44% ($p<0.001$)), for the SUDHL10 cell line (RS), a concentration of 1 μ M olaparib with rituximab decreased cell viability from 105% to 63% ($p<0.001$), for the U2932 cell line (RI), cell viability decreased from 60% to 53% at 5 μ M ($p=0.003$), and for the SC-1 cell line (RI), a concentration of 10 μ M olaparib plus rituximab decreased cell viability from 33% to 26% ($p=0.03$) (Figure 4C). In conclusion, the combination of WEE1 or PARP1 inhibition with rituximab resulted in enhanced cytotoxicity and reduced cell viability in 3 out of 4 tested almost all DLBCL cell lines. The added effect of the WEE1 or PARP1 inhibitors with rituximab was independent of rituximab sensitivity.

Discussion

In this study, we performed a large meta-analysis on the transcriptomic data of 1,804 DLBCL patient samples to identify drug-target combinations for improvement of standard DLBCL immunochemotherapy (R-CHOP). We therefore took CD20, which is part of the BCR signalosome and a key target in DLBCL treatment, as the central protein to perform a guilt-by-association analysis. By employing CD20 for guilt-by-association we aimed to find targets with similar expression patterns to CD20. We focused on the associated genes as therapeutic targets for DLBCL. Co-expression does not necessarily indicate a direct relation or interaction with CD20, but was used for selection of promising targets. Guilt-by-association analysis has been used in cancer research to identify biomarkers. However, as a therapeutic purpose, guilt-by-association has been used only to identify targets in defined pathways, such as cancer metabolism [28]. In the present study, we used this method for the first time to identify targets in relation to a single gene—CD20—which is a central molecule for current treatment regimens of DLBCL patients. This guilt-by-association approach may also be applied more generally in future studies to improve drug combinations for other types of

cancer and any starting gene with a central role in standard therapies.

We selected the top 500 associated probes, corresponding to 390 protein-encoding MS4A1-associated genes. All well-known genes to be actively involved and expressed in DLBCL were present, including for instance BTK as a target for ibrutinib in current DLBCL clinical trials. In addition, we identified many genes for which the pathogenetic relevance in the context of DLBCL is still unknown (Table 2). From this list, candidate drug-targets were selected when not involved in BCR signaling or currently already under clinical study in DLBCL. Moreover, only clinical-grade inhibiting drugs from the treatment of other (solid) malignancies were selected to accelerate their application in clinical trials. The choice for clinically approved drugs also circumvents the problem of a worldwide lack of a proper mouse model to study the effect of rituximab *in vivo*. The human Fc region of the chimeric IgG1 antibody rituximab lacks the ability to activate the murine complement (CDC) and effector cells (ADCC) [29,30], thereby limiting the study of relevant rituximab-drug combinations in a murine or xenogeneic setting.

Our selection revealed multiple targets which were more strongly associated with CD20 than other well-known targets in DLBCL. We therefore consider them to be of high potential for direct combination with current DLBCL treatment. Examples are CDK1 (cell cycle; targeted by dinaciclib, PRKD3 (signaling protein; targeted by momelotinib), WEE1 (replication checkpoint kinase; targeted by AZD1775) and PARP1 (DNA repair; targeted by olaparib).

For primary investigation we chose WEE1 and PARP1. Although neither of these genes have been investigated in DLBCL in combination with rituximab, both WEE1 and PARP1 have clinically approved inhibiting drugs and have been studied extensively *in vivo*. Both are currently used in clinical trials for several (solid) cancers, including cervical cancer, ovarian cancer, breast cancer, lung cancer, adenocarcinoma and gliomas (ClinicalTrials.gov). Another important reason for our interest in these two genes was based on DLBCL pathophysiology. DLBCL originates from normal B-cells due to aberrant effects of somatic hypermutation and class-switch recombination machinery during the germinal center reaction, which results in chromosomal breaks leading to oncogenic transformation of B cells [31,32]. There is a crucial role for DNA damage response (DDR) and repair proteins during the germinal center reaction [33] and high expression of DNA damage response proteins have been demonstrated in DLBCL patient cases [34]. Since DLBCL is a tumor with high levels of DNA damage, targeting proteins involved in DDR and damage repair, such as WEE1 and PARP1, is a rational choice for therapy in DLBCL.

WEE1 is a replication checkpoint kinase that prevents the onset of mitosis in cells that have incompletely replicated or have damaged genomes. In case of DNA damage, WEE1 indirectly arrests the cells at the G2/M checkpoint, allowing time for repair or resulting in cell death [35]. Targeting WEE1 with AZD1775 in patients with a diversity of chemorefractory solid tumors demonstrated single agent activity [36]. Targeting WEE1 with AZD1775 in combination with the CHK1 inhibitor PF-00477736 resulted in cell killing and destabilization of the oncogenic transcription factor MYC in DLBCL and was strongly synergistic in mantle cell lymphoma [37,38]. Moreover, great potential has been shown for WEE1 inhibition in combination with cell cycle arresting chemotherapeutics such as doxorubicin and cytarabine

[39]. Our results show that WEE1 is highly expressed in DLBCL patient specimen. In addition, we demonstrated that the combination of the WEE1 inhibitor AZD1775 and rituximab resulted in additive cytotoxicity for all tested DLBCL cell lines, also in the rituximab-insensitive cell lines.

PARP1 is well-known for its role in repairing DNA single strand breaks, and is thought to accumulate at sites of damage, inducing chromatin remodeling and attracting DNA repair factors [40]. PARP inhibitors have been mainly used in a setting of defective double strand break repair (DSBR), as PARP inhibition leads to double stranded breaks, which causes synthetic lethality in a DSB repair defective background. To this extent, PARP1 inhibition has proven to be successful when used in DDR deficient tumors such as BRCA1- or BRCA2-deficient breast cancer, ATM-deficient colorectal cancer [41], ATM-deficient lung cancer [42], TP53/ATM-deficient MCL [43], IGH/MYC-induced BRCA2 deficient Burkitt lymphoma [44] and PTEN/ TP53-deficient prostate cancer [45]. In DLBCL, TP53 mutations are found in 21–24% of cases and are inversely correlated with survival [46,47]. Moreover, PARP1 is known for its role in NF- κ B activation [48] contributing to inflammation and carcinogenesis. Therefore, targeting PARP1 in a setting of high genomic instability, as seen in DLBCL, and high NF- κ B activation, as seen in the ABC type DLBCL [49], is an understandable choice. Our results demonstrate that PARP1 is highly expressed in DLBCL patient samples. Interestingly, this finding is supported by the recently published PARP1-targeted PET imaging approach which can differentiate malignant from inflamed lymph nodes in DLBCL [50]. The combination of the PARP inhibitor olaparib and rituximab enhanced cytotoxicity in all 4 DLBCL cell lines tested, which all carried mutations in the TP53 gene. Consequently, combining PARP1 inhibitors with current therapy could improve survival of patients with mutant TP53. Recently, the potential synergistic effects of combining WEE1 and PARP1 inhibition in acute leukemia revealed also a potential synergistic effect, creating a double-hit model by increasing DNA damage and preventing DNA damage repair [51].

A potential bias of our approach might have been the selection of only high-quality mRNA samples. For this reason, we performed survival analyses for the different COO DLBCL groups and for CHOP versus R-CHOP treated DLBCL patients. These results were similar to survival data as reported in the literature. The addition of rituximab to CHOP chemotherapy seems more beneficial in GCB-DLBCL with high WEE1 expression compared to low WEE1 expression. This might be explained by the correlation of WEE1 with CD20 expression level as observed in our guilt-by-association analysis, as patients with low CD20 expression also have inferior survival [30,52]. For PARP1, our data showed that patients with a relatively high PARP1 expression in ABC-DLBCL benefitted the most from the addition of rituximab to CHOP chemotherapy. This suggests an additional effect of PARP1 response in the ABC subtype patients to rituximab. We hypothesize that this might be explained by the continuous activation and essential role of NF- κ B in ABC-subtype DLBCL. Rituximab directly inhibits subunits of the NF- κ B pathway [53] and might therefore lead to accumulation of more damage in ABC-type DLBCL that depends on high PARP1 expression for repair and NF- κ B activation.

In conclusion, a genome wide analysis of MS4A1 (CD20) guilt-by-association and drug-target prioritization has been able to identify potentially relevant drug-targets to combine with

and improve DLBCL treatment. For the identified genes WEE1 and PARP1 clinically approved inhibitory drugs showed improved DLBCL cell killing when combined with rituximab. Our approach may be used as a fast-track approach to direct the use of clinically approved agents in future phase I/II trials to improve standard DLBCL treatment.

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Supporting information

Supplemental Table 1. GSE accession numbers. Series identifier and GPL platform numbers that were used in this study, including corresponding references.

Series identifier	Number of included patient samples	Platform	COO	Age	Gender	Treatment	Respons	MYC	References
GSE10172	36	GPL96	yes	yes	yes	no	no	yes	18509088, 23143595
GSE10524	15	GPL570	no	no	yes	no	no	no	citation missing
GSE10846	420	GPL570	yes	yes	yes	yes	yes	no	19038878, 21546504
GSE11318	10	GPL570	yes	no	yes	no	yes	no	18765795
GSE12195	73	GPL570	yes/no	no	no	no	no	no	22137796, 21390126, 21156281, 19965633, 19412164
GSE12453	10	GPL570	no	no	no	no	no	no	18794340, 24376854
GSE12630	2	GPL96	no	no	yes	no	no	no	19332734
GSE19246	59	GPL570	yes	no	no	no	no	no	20688907
GSE22470	270	GPL96	yes	yes	yes	no	no	no	21487109, 22238326, 23143595
GSE23501	26	GPL570	yes	yes	yes	yes	yes	no	20610814
GSE25639	26	GPL570	no	no	no	no	no	no	22689981
GSE31312	364	GPL570	yes	no	no	yes	no	no	26324762, 26248897, 26111978, 22437443, 23982177, 23775435
GSE34171	164	GPL570 + GPL96	yes	no	no	no	no	no	22975378, 2376004
GSE38885	21	GPL570	no	no	no	no	no	no	23489474
GSE44164	32	GPL96	yes	yes	yes	no	no	yes	24179151
GSE44337	9	GPL570	no	no	no	no	no	no	citation missing

Supplemental Table 1 continued.

Series identifier	Number of included patient samples	Platform	COO	Age	Gender	Treatment	Respons	MYC	References
GSE4475	220	GPL96	yes	yes	yes	yes	yes	yes	16760442, 23143595
GSE48097 / GSE48184	29	GPL570	yes	no	no	no	no	no	24030260
GSE57611 / GSE57612	11	GPL96	yes	no	no	no	no	no	25042405
GSE9429	7	GPL96	no	no	no	no	no	no	19373655

Supplemental Table 2. CD20 guilt-by-association. Shown are the first 50 MS4A1-associated gene probes with corresponding gene symbol, gene title and chromosomal location by Pearson's correlation with confidence intervals (CI). MVP reject H0 (FDR 1%, CI 99%, nPerm 1000) is true for all probes reported in this table.

No.	Probe	Gene Symbol	Gene Title	Chromosomal Location	Pearson's Correlation	Lower 95% CI R	Upper 95% CI R	Z	Sig. (2-tailed)
1	209307_at	SWAP70	SWAP switching B-cell complex subunit	chr11p15	0,55	0,52	0,58	25,92	4,15E-148
2	209306_s_at	SWAP70	SWAP switching B-cell complex subunit	chr11p15	0,54	0,51	0,58	25,48	3,04E-143
3	221969_at	PAX5	paired box 5	chr9p13	0,54	0,50	0,57	25,09	7,15E-139
4	219497_s_at	BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	chr2p16.1	0,53	0,49	0,56	24,39	2,12E-131
5	219498_s_at	BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	chr2p16.1	0,52	0,48	0,55	24,05	8,21E-128
6	205049_s_at	CD79A	CD79a molecule, immunoglobulin-associated alpha	chr19q13.2	0,48	0,45	0,52	22,04	1,08E-107
7	201259_s_at	SYPL1	synaptophysin-like 1	chr7q22.3	0,48	0,45	0,52	22,00	2,77E-107
8	216899_s_at	SKAP2	src kinase associated phosphoprotein 2	chr7p15.2	0,47	0,43	0,50	21,22	6,21E-100
9	205801_s_at	RASGRP3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	chr2p25.1-p24.1	0,45	0,41	0,48	19,98	7,86E-89
10	217422_s_at	CD22	CD22 molecule	chr19q13.1	0,44	0,40	0,48	19,72	1,43E-86
11	218236_s_at	PRKD3	protein kinase D3	chr2p21	0,44	0,40	0,47	19,49	1,38E-84
12	204581_at	CD22	CD22 molecule	chr19q13.1	0,43	0,39	0,47	19,34	2,71E-83
13	221781_s_at	DNAJC10	DnaJ (Hsp40) homo-log, subfamily C, member 10	chr2q32.1	0,43	0,39	0,46	19,02	1,12E-80
14	219518_s_at	ELL3	elongation factor RNA polymerase II-like 3	chr15q15.3	0,43	0,39	0,46	19,02	1,18E-80
15	218640_s_at	PLEKHF2	pleckstrin homology domain containing, family F (with FYVE domain) member 2	chr8q22.1	0,43	0,39	0,46	18,94	5,33E-80
16	210347_s_at	BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	chr2p16.1	0,42	0,38	0,46	18,90	1,21E-79
17	38521_at	CD22	CD22 molecule	chr19q13.1	0,42	0,38	0,46	18,79	9,95E-79

Supplemental Table 2 continued.

No.	Probe	Gene Symbol	Gene Title	Chromosomal Location	Pearson's Correlation	Lower 95% CI R	Upper 95% CI R	Z	Sig. (2-tailed)
18	204361_s_at	SKAP2	src kinase associated phosphoprotein 2	chr7p15.2	0.42	0.38	0.46	18,78	1,06E-78
19	208456_s_at	RRAS2	related RAS viral (r-ras) oncogene homolog 2	chr11p15.2	0.42	0.38	0.46	18,60	2,95E-77
20	216250_s_at	LPXN	leupaxin	chr11q12.1	0.41	0.37	0.45	18,39	1,60E-75
21	202374_s_at	AURKAPS1 /// RAB3GAP2	aurora kinase A pseudogene 1 /// RAB3 GTPase activating protein subunit 2 (non-catalytic)	chr1q41	0.40	0.36	0.44	17,91	9,11E-72
22	204674_at	LRMP	lymphoid-restricted membrane protein	chr12p12.1	0.40	0.36	0.44	17,85	3,07E-71
23	211352_s_at	NCOA3	nuclear receptor coactivator 3	chr20q12	0.40	0.36	0.44	17,82	5,03E-71
24	204192_at	CD37	CD37 molecule	chr19q13.3	0.40	0.36	0.44	17,66	8,80E-70
25	201260_s_at	SYPL1	synaptophysin-like 1	chr7q22.3	0.40	0.36	0.44	17,57	4,02E-69
26	205297_s_at	CD79B	CD79b molecule, immunoglobulin-associated beta	chr17q23	0.40	0.36	0.44	17,57	4,12E-69
27	35974_at	LRMP	lymphoid-restricted membrane protein	chr12p12.1	0.40	0.36	0.44	17,53	8,90E-69
28	209580_s_at	MBD4	methyl-CpG binding domain protein 4	chr3q21.3	0.40	0.36	0.44	17,51	1,18E-68
29	215711_s_at	WEE1	WEE1 homolog (S. pombe)	chr11p15.3-p15.1	0.40	0.36	0.44	17,51	1,31E-68
30	203185_at	RASSF2	Ras association (RalGDS/AF-6) domain family member 2	chr20p13	0.40	0.36	0.44	17,50	1,33E-68
31	201299_s_at	MOB1A	MOB kinase activator 1A	chr2p13.1	0.40	0.35	0.43	17,45	3,58E-68
32	200008_s_at	GDI2	GDP dissociation inhibitor 2	chr10p15	0.39	0.35	0.43	17,28	6,74E-67
33	206206_at	CD180	CD180 molecule	chr5q12	0.39	0.35	0.43	17,27	8,31E-67
34	206398_s_at	CD19	CD19 molecule	chr16p11.2	0.39	0.35	0.43	17,23	1,57E-66
35	201720_s_at	LAPTM5	lysosomal protein transmembrane 5	chr1p34	0.39	0.35	0.43	17,17	4,53E-66

Supplemental Table 2 continued.

No.	Probe	Gene Symbol	Gene Title	Chromosomal Location	Pearson's Correlation	Lower 95% CI R	Upper 95% CI R	Z	Sig. (2-tailed)
36	214190_x_at	GGA2	golgi-associated, gamma adaptin ear containing, ARF-binding protein 2	chr16p12	0,39	0,35	0,43	17,15	5,88E-66
37	208621_s_at	EZR	ezrin	chr6q25.3	0,39	0,35	0,43	17,15	5,89E-66
38	210658_s_at	GGA2	golgi-associated, gamma adaptin ear containing, ARF-binding protein 2	chr16p12	0,39	0,35	0,43	17,09	1,92E-65
39	213548_s_at	ODV3	CDV3 homolog (mouse)	chr3q22.1	0,39	0,35	0,43	17,06	2,95E-65
40	211559_s_at	CCNG2	cyclin G2	chr4q21.1	0,39	0,35	0,43	17,06	3,20E-65
41	219517_at	ELL3	elongation factor RNA polymerase II-like 3	chr15q15.3	0,39	0,35	0,43	17,05	3,64E-65
42	206860_s_at	MIOS	missing oocyte, meiosis regulator, homolog (Drosophila)	chr7p21.3	0,39	0,35	0,43	17,00	8,66E-65
43	217234_s_at	EZR	ezrin	chr6q25.3	0,38	0,34	0,42	16,92	3,38E-64
44	204215_at	TMEM243	transmembrane protein 243, mitochondrial	chr7q21.12	0,38	0,34	0,42	16,82	1,67E-63
45	212590_at	RRAS2	related RAS viral (r-ras) oncogene homolog 2	chr11p15.2	0,38	0,34	0,42	16,79	2,69E-63
46	204362_at	SKAP2	src kinase associated phosphoprotein 2	chr7p15.2	0,38	0,34	0,42	16,68	1,82E-62
47	220059_at	STAP1	signal transducing adaptor family member 1	chr4q13.2	0,38	0,34	0,42	16,67	2,07E-62
48	205153_s_at	CD40	CD40 molecule, TNF receptor superfamily member 5	chr20q12-q13.2	0,38	0,34	0,42	16,61	5,90E-62
49	221586_s_at	E2F5	E2F transcription factor 5, p130-binding	chr8q21.2	0,38	0,34	0,42	16,59	8,65E-62
50	217811_at	SELT	selenoprotein T	chr3q25.1	0,38	0,33	0,42	16,48	5,02E-61

Supplemental Table 3A. Table enrichment analysis: KEGG database.

Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	B cell receptor signaling pathway_Homo sapiens_hsa04662	7,70E-09	1,69E-06	-2,00	40,35
2	Fc gamma R-mediated phagocytosis_Homo sapiens_hsa04666	2,71E-05	2,97E-06	-1,83	23,32
3	Ribosome_Homo sapiens_hsa03010	6,71E-05	4,90E-06	-1,70	20,76
4	RNA transport_Homo sapiens_hsa03013	1,30E-06	7,14E-05	-1,79	17,13
5	NF-kappa B signaling pathway_Homo sapiens_hsa04064	1,78E-06	7,78E-05	-1,65	15,64
6	Epstein-Barr virus infection_Homo sapiens_hsa05169	4,03E-05	1,10E-03	-1,78	12,09
7	Mismatch repair_Homo sapiens_hsa03430	3,80E-06	1,39E-04	-1,24	11,00
8	Transcriptional misregulation in cancer_Homo sapiens_hsa05202	4,95E-05	1,21E-03	-1,56	10,49
9	Shigellosis_Homo sapiens_hsa05131	3,46E-05	1,08E-03	-1,53	10,42
10	mRNA surveillance pathway_Homo sapiens_hsa03015	6,58E-05	1,39E-03	-1,48	9,71
11	Pathogenic Escherichia coli infection_Homo sapiens_hsa05130	8,63E-05	1,58E-03	-1,40	9,05
12	Regulation of actin cytoskeleton_Homo sapiens_hsa04810	2,81E-04	4,39E-03	-1,60	8,68
13	Cell cycle_Homo sapiens_hsa04110	1,48E-04	2,49E-03	-1,43	8,57
14	Fc epsilon RI signaling pathway_Homo sapiens_hsa04664	3,33E-04	4,56E-03	-1,56	8,41
15	Primary immunodeficiency_Homo sapiens_hsa05340	6,97E-05	1,39E-03	-1,20	7,92
16	Platelet activation_Homo sapiens_hsa04611	6,07E-04	7,39E-03	-1,47	7,20
17	Ubiquitin mediated proteolysis_Homo sapiens_hsa04120	3,33E-04	4,56E-03	-1,29	6,94
18	Pyrimidine metabolism_Homo sapiens_hsa00240	9,80E-04	1,13E-02	-1,30	5,82
19	Oocyte meiosis_Homo sapiens_hsa04114	2,69E-03	2,94E-02	-1,38	4,87
20	Endocytosis_Homo sapiens_hsa04144	4,68E-03	4,88E-02	-1,47	4,44
21	Viral carcinogenesis_Homo sapiens_hsa05203	6,67E-03	6,01E-02	-1,40	3,93
22	Base excision repair_Homo sapiens_hsa03410	3,97E-04	5,11E-03	-0,67	3,51
23	Pathways in cancer_Homo sapiens_hsa05200	1,07E-02	8,06E-02	-1,36	3,43
24	Purine metabolism_Homo sapiens_hsa00230	7,32E-03	6,17E-02	-1,18	3,30
25	Tuberculosis_Homo sapiens_hsa05152	7,86E-03	6,37E-02	-1,17	3,22

Supplemental Table 3B. Table enrichment analysis: Wiki pathways.

Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	B Cell Receptor Signaling Pathway_Homo sapiens_WP23	1,81E-18	4,85E-16	-2,07	87,26
2	Cytoplasmic Ribosomal Proteins_Homo sapiens_WP477	1,53E-07	1,61E-05	-1,98	35,57
3	Retinoblastoma (RB) in Cancer_Homo sapiens_WP2446	1,80E-07	1,61E-05	-1,90	34,05
4	Cytoplasmic Ribosomal Proteins_Mus musculus_WP163	7,40E-07	4,96E-05	-1,94	32,64
5	Translation Factors_Homo sapiens_WP107	5,03E-04	2,70E-05	-1,93	20,29
6	mRNA processing_Mus musculus_WP310	9,59E-04	4,28E-05	-2,01	20,21
7	Translation Factors_Mus musculus_WP307	2,46E-06	9,42E-05	-1,75	16,23
8	G1 to S cell cycle control_Homo sapiens_WP45	6,11E-06	2,05E-04	-1,84	15,63
9	G1 to S cell cycle control_Mus musculus_WP413	1,91E-05	5,68E-04	-1,77	13,26
10	XPodNet - protein-protein interactions in the podocyte expanded by STRING_Mus musculus_WP2309	1,04E-04	2,08E-03	-1,93	11,94
11	Interferon type I signaling pathways_Homo sapiens_WP585	7,66E-05	2,05E-03	-1,81	11,20
12	IL-3 Signaling Pathway_Mus musculus_WP373	1,08E-04	2,08E-03	-1,77	10,92
13	TNF-alpha NF-kB Signaling Pathway_Mus musculus_WP246	1,94E-04	3,46E-03	-1,90	10,78
14	IL-6 signaling Pathway_Mus musculus_WP387	1,08E-04	2,08E-03	-1,73	10,66
15	Pathogenic Escherichia coli infection_Homo sapiens_WP2272	8,63E-05	2,08E-03	-1,64	10,13
16	EGF/EGFR Signaling Pathway_Homo sapiens_WP437	3,36E-04	5,62E-03	-1,69	8,77
17	TNF alpha Signaling Pathway_Homo sapiens_WP231	4,06E-04	6,40E-03	-1,52	7,69
18	Integrated Breast Cancer Pathway_Homo sapiens_WP1984	8,78E-04	1,07E-02	-1,61	7,29
19	mRNA Processing_Homo sapiens_WP411	8,11E-04	1,07E-02	-1,46	6,63
20	Cell Cycle_Homo sapiens_WP179	8,64E-04	1,07E-02	-1,37	6,24
21	EGFR1 Signaling Pathway_Mus musculus_WP572	1,84E-03	1,97E-02	-1,56	6,11
22	TCR Signaling Pathway_Homo sapiens_WP69	1,79E-03	1,97E-02	-1,41	5,53
23	IL-5 Signaling Pathway_Homo sapiens_WP127	9,85E-04	1,15E-02	-1,21	5,42
24	IL-5 Signaling Pathway_Mus musculus_WP151	1,99E-03	2,04E-02	-1,34	5,20
25	Regulation of Actin Cytoskeleton_Homo sapiens_WP51	2,58E-03	2,29E-02	-1,37	5,19

Supplemental Table 3C. Table enrichment analysis: Biocarta.

Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	BCR Signaling Pathway_Homo sapiens_h_bcrPathway	1,67E-06	1,22E-04	-1,90	17,11
2	How does salmonella hijack a cell_Homo sapiens_h_salmonellaPathway	1,10E-06	1,22E-04	-1,03	9,32
3	Role of MAL in Rho-Mediated Activation of SRF_Homo sapiens_h_malPathway	2,43E-05	7,09E-04	-1,23	8,91
4	Y branching of actin filaments_Homo sapiens_h_actinYPathway	9,57E-06	3,49E-04	-1,09	8,69
5	Fc Epsilon Receptor I Signaling in Mast Cells_Homo sapiens_h_fcer1Pathway	2,12E-04	4,41E-03	-1,42	7,72
6	Human Cytomegalovirus and Map Kinase Pathways_Homo sapiens_h_hcmvPathway	2,07E-04	4,41E-03	-1,41	7,66
7	Role of PI3K subunit p85 in regulation of Actin Organization and Cell Migration_Homo sapiens_h_cdc42racPathway	9,57E-06	3,49E-04	-0,94	7,50
8	Cell Cycle: G1/S Check Point_Homo sapiens_h_g1Pathway	1,46E-03	2,66E-02	-1,01	3,68
9	p38 MAPK Signaling Pathway_Homo sapiens_h_p38mapkPathway	2,52E-03	3,68E-02	-1,08	3,58
10	MAPKinase Signaling Pathway_Homo sapiens_h_mapkPathway	4,45E-03	5,90E-02	-0,97	2,74
11	Role of Erk5 in Neuronal Survival_Homo sapiens_h_erk5Pathway	1,34E-02	1,03E-01	-0,73	1,65
12	Regulation of eIF4e and p70 S6 Kinase_Homo sapiens_h_eif4Pathway	1,07E-02	9,92E-02	-0,67	1,55
13	Skeletal muscle hypertrophy is regulated via AKT/mTOR pathway_Homo sapiens_h_igf1mtorpathway	1,20E-02	9,92E-02	-0,54	1,25
14	Keratinocyte Differentiation_Homo sapiens_h_keratinocytePathway	1,90E-02	1,25E-01	-0,56	1,16
15	FAS signaling pathway (CD95)_Homo sapiens_h_fasPathway	6,37E-03	7,64E-02	-0,45	1,16
16	RB Tumor Suppressor/Checkpoint Signaling in response to DNA damage_Homo sapiens_h_rbPathway	1,77E-03	2,87E-02	-0,32	1,14
17	Angiotensin II mediated activation of JNK Pathway via Pyk2 dependent signaling_Homo sapiens_h_At1rPathway	2,74E-02	1,37E-01	-0,46	0,91
18	Phosphoinositides and their downstream targets,_Homo sapiens_h_ptdinsPathway	7,33E-03	7,64E-02	-0,26	0,66
19	HIV-1 Nef: negative effector of Fas and TNF_Homo sapiens_h_HivnefPathway	1,90E-02	1,25E-01	-0,26	0,54
20	Erk and PI-3 Kinase Are Necessary for Collagen Binding in Corneal Epithelia_Homo sapiens_h_ecmPathway	2,15E-02	1,26E-01	-0,23	0,47
21	WNT Signaling Pathway_Homo sapiens_h_wntPathway	1,97E-02	1,25E-01	-0,09	0,18
22	Ceramide Signaling Pathway_Homo sapiens_h_ceramidePathway	2,54E-02	1,37E-01	-0,07	0,14

Supplemental Table 3D. Table enrichment analysis: NCI Nature.

Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	BCR signaling pathway_Homo sapiens_acbf44e2-618c-11e5-8ac5-06603eb7f303	3,04E-12	4,96E-10	-1,84	52,00
2	ErbB1 downstream signaling_Homo sapiens_30d60550-6192-11e5-8ac5-06603eb7f303	1,78E-07	1,45E-05	-1,77	31,86
3	PDGFR-beta signaling pathway_Homo sapiens_c901a3e4-6194-11e5-8ac5-06603eb7f303	2,67E-05	1,45E-06	-1,96	26,31
4	RAC1 signaling pathway_Homo sapiens_faffa4fc-6194-11e5-8ac5-06603eb7f303	8,58E-06	3,50E-04	-1,65	13,15
5	CDC42 signaling events_Homo sapiens_50b98ae0-6190-11e5-8ac5-06603eb7f303	5,95E-05	1,94E-03	-1,65	10,30
6	E2F transcription factor network_Homo sapiens_bb4d0fd3-6191-11e5-8ac5-06603eb7f303	7,30E-05	1,98E-03	-1,54	9,60
7	Class I PI3K signaling events_Homo sapiens_12b82bb7-6191-11e5-8ac5-06603eb7f303	3,08E-04	7,16E-03	-1,36	6,74
8	Fc-epsilon receptor I signaling in mast cells_Homo sapiens_86cd7795-6192-11e5-8ac5-06603eb7f303	8,63E-04	1,28E-02	-1,28	5,60
9	IL8- and CXCR2-mediated signaling events_Homo sapiens_fe78e284-6193-11e5-8ac5-06603eb7f303	4,58E-04	9,33E-03	-1,16	5,40
10	GMCSF-mediated signaling events_Homo sapiens_095aa3ef-6193-11e5-8ac5-06603eb7f303	6,01E-04	1,09E-02	-1,15	5,19
11	FAS (CD95) signaling pathway_Homo sapiens_79cc9c14-6192-11e5-8ac5-06603eb7f303	7,76E-04	1,26E-02	-1,14	5,00
12	C-MYB transcription factor network_Homo sapiens_61020228-618e-11e5-8ac5-06603eb7f303	1,04E-03	1,41E-02	-1,12	4,77
13	Ceramide signaling pathway_Homo sapiens_d3747df2-6190-11e5-8ac5-06603eb7f303	1,53E-03	1,91E-02	-0,91	3,61
14	Integrin-linked kinase signaling_Homo sapiens_21738158-6194-11e5-8ac5-06603eb7f303	1,69E-03	1,97E-02	-0,87	3,40
15	TNF receptor signaling pathway_Homo sapiens_316be05e-6196-11e5-8ac5-06603eb7f303	1,87E-03	1,98E-02	-0,80	3,13
16	p73 transcription factor network_Homo sapiens_a88c505e-6194-11e5-8ac5-06603eb7f303	3,97E-03	3,41E-02	-0,84	2,84
17	Thromboxane A2 receptor signaling_Homo sapiens_27d5800d-6196-11e5-8ac5-06603eb7f303	4,45E-03	3,63E-02	-0,83	2,76
18	Regulation of retinoblastoma protein_Homo sapiens_407a3468-6195-11e5-8ac5-06603eb7f303	7,84E-03	5,14E-02	-0,65	1,94
19	Regulation of Telomerase_Homo sapiens_4dfe97ca-6195-11e5-8ac5-06603eb7f303	9,48E-03	5,94E-02	-0,56	1,59
20	Alpha-synuclein signaling_Homo sapiens_588134bc-6187-11e5-8ac5-06603eb7f303	3,20E-03	2,90E-02	-0,42	1,48
21	Signaling events mediated by Hepatocyte Growth Factor Receptor (c-Met)_Homo sapiens_ac39d2b9-6195-11e5-8ac5-06603eb7f303	1,66E-02	9,02E-02	-0,59	1,41
22	Retinoic acid receptors-mediated signaling_Homo sapiens_5797691b-6195-11e5-8ac5-06603eb7f303	1,94E-03	1,98E-02	-0,33	1,30
23	Internalization of ErbB1_Homo sapiens_3aa9aafa-6194-11e5-8ac5-06603eb7f303	7,22E-03	5,12E-02	-0,43	1,27
24	amb2 Integrin signaling_Homo sapiens_5d4f90b6-6188-11e5-8ac5-06603eb7f303	7,22E-03	5,12E-02	-0,26	0,77
25	Noncanonical Wnt signaling pathway_Homo sapiens_7ff5fe76-6194-11e5-8ac5-06603eb7f303	3,20E-03	2,90E-02	-0,20	0,69

Supplemental Table 3E. Table enrichment analysis: Panther.

Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	B cell activation_Homo sapiens_P00010	4,79E-08	3,78E-06	-1,69	32,75
2	Inflammation mediated by chemokine and cytokine signaling pathway_Homo sapiens_P00031	3,66E-03	5,16E-02	-1,53	4,52
3	Apoptosis signaling pathway_Homo sapiens_P00006	3,63E-03	5,16E-02	-1,49	4,43
4	Cytoskeletal regulation by Rho GTPase_Homo sapiens_P00016	2,30E-03	5,16E-02	-1,36	4,02
5	VEGF signaling pathway_Homo sapiens_P00056	3,80E-03	5,16E-02	-1,34	3,97
6	CCKR signaling map ST_Homo sapiens_P06959	4,85E-03	5,16E-02	-1,23	3,65
7	EGF receptor signaling pathway_Homo sapiens_P00018	5,23E-03	5,16E-02	-1,08	3,20
8	Parkinson disease_Homo sapiens_P00049	4,78E-03	5,16E-02	-1,02	3,03
9	T cell activation_Homo sapiens_P00053	1,34E-02	8,84E-02	-0,51	1,23
10	PDGF signaling pathway_Homo sapiens_P00047	2,15E-02	1,31E-01	-0,50	1,03
11	Ubiquitin proteasome pathway_Homo sapiens_P00060	9,32E-03	7,36E-02	-0,34	0,90
12	FGF signaling pathway_Homo sapiens_P00021	4,29E-02	1,95E-01	-0,05	0,08
13	Ras Pathway_Homo sapiens_P04393	4,43E-02	1,95E-01	0,09	-0,14
14	Angiotensin II-stimulated signaling through G proteins and beta-arrestin_Homo sapiens_P05911	2,74E-02	1,55E-01	0,13	-0,23
15	Cadherin signaling pathway_Homo sapiens_P00012	9,47E-01	9,47E-01	5,59	-0,31
16	Huntington disease_Homo sapiens_P00029	9,24E-02	3,06E-01	0,37	-0,44
17	Alzheimer disease-presenilin pathway_Homo sapiens_P00004	8,56E-01	8,66E-01	6,36	-0,91
18	Angiogenesis_Homo sapiens_P00005	1,41E-01	3,83E-01	1,02	-0,98
19	Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway_Homo sapiens_P00027	1,49E-01	3,92E-01	1,10	-1,03
20	Interleukin signaling pathway_Homo sapiens_P00036	8,14E-01	8,35E-01	5,72	-1,03
21	p53 pathway by glucose deprivation_Homo sapiens_P04397	7,33E-03	6,43E-02	0,39	-1,07
22	p53 pathway_Homo sapiens_P00059	1,58E-01	3,94E-01	1,52	-1,42
23	Nicotinic acetylcholine receptor signaling pathway_Homo sapiens_P00044	7,35E-01	7,64E-01	5,97	-1,61
24	Adrenaline and noradrenaline biosynthesis_Homo sapiens_P00001	8,33E-02	3,06E-01	1,56	-1,85
25	Alpha adrenergic receptor signaling pathway_Homo sapiens_P00002	7,20E-02	3,00E-01	1,54	-1,86

Supplemental Table 3F. Table enrichment analysis: GO biological process.

<i>Index</i>	<i>Name</i>	<i>P-value</i>	<i>Adjusted p-value</i>	<i>Z-score</i>	<i>Combined score</i>
1	B cell activation (GO:0042113)	8,73E-19	2,40E-15	-2,18	88,40
2	gene expression (GO:0010467)	1,09E-12	1,50E-09	-2,34	63,63
3	immune response-activating signal transduction (GO:0002757)	1,42E-07	2,29E-05	-3,55	62,46
4	lymphocyte activation (GO:0046649)	6,53E-12	4,48E-09	-2,32	60,66
5	B cell receptor signaling pathway (GO:0050853)	1,71E-11	9,36E-09	-2,36	59,95
6	immune response-activating cell surface receptor signaling pathway (GO:0002429)	1,01E-06	1,21E-04	-3,68	58,66
7	translational initiation (GO:0006413)	2,74E-12	2,50E-09	-2,18	58,28
8	leukocyte activation (GO:0045321)	6,42E-11	2,94E-08	-2,37	57,40
9	activation of immune response (GO:0002253)	4,52E-07	6,20E-05	-3,40	56,49
10	immune response-regulating cell surface receptor signaling pathway (GO:0002768)	3,09E-06	3,39E-04	-3,51	52,25
11	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay (GO:0000184)	9,68E-11	3,80E-08	-2,13	51,03
12	nuclear-transcribed mRNA catabolic process (GO:0000956)	8,79E-09	2,68E-06	-2,20	43,36
13	translation (GO:0006412)	6,56E-08	1,20E-05	-2,33	42,49
14	translational elongation (GO:0006414)	6,88E-09	2,36E-06	-2,11	41,98
15	mRNA catabolic process (GO:0006402)	2,55E-08	6,37E-06	-2,22	41,94
16	macromolecular complex disassembly (GO:0032984)	3,59E-08	8,20E-06	-2,19	40,77
17	translational termination (GO:0006415)	1,33E-08	3,66E-06	-2,09	40,54
18	protein complex disassembly (GO:0043241)	8,59E-08	1,48E-05	-2,17	39,15
19	cellular protein complex disassembly (GO:0043624)	5,89E-08	1,20E-05	-2,12	38,68
20	viral transcription (GO:0019083)	6,47E-08	1,20E-05	-2,07	37,74
21	RNA catabolic process (GO:0006401)	3,31E-07	5,05E-05	-2,23	37,39
22	antigen receptor-mediated signaling pathway (GO:0050851)	3,88E-07	5,61E-05	-2,21	36,97
23	protein localization to organelle (GO:0033365)	9,13E-07	1,14E-04	-2,31	36,95
24	Fc receptor signaling pathway (GO:0038093)	1,52E-06	8,56E-05	-3,63	34,02
25	establishment of protein localization to endoplasmic reticulum (GO:0072599)	7,16E-07	9,37E-05	-2,06	33,28

Supplemental Table 4. CD20 guilt-by-association subgroup analysis. The first 500 MS4A1-associated gene probes with corresponding gene name were grouped according to biological pathway.

Gene	Name	Chromosome	Pearson	P	Biological pathway
SWAP70	SWAP switching B-cell complex 70kDa subunit	chr11p15	0,55	4,15E-148	BCR signaling
PAX5	paired box 5	chr9p13	0,54	7,15E-139	BCR signaling
BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	chr2p16.1	0,53	2,12E-131	BCR signaling
CD79A	CD79a molecule, immunoglobulin-associated alpha	chr19q13.2	0,48	1,08E-107	BCR signaling
SKAP2	src kinase associated phosphoprotein 2	chr7p15.2	0,47	6,21E-100	BCR signaling
RASGRP3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	chr2p25.1-p24.1	0,45	7,86E-89	BCR signaling
CD22	CD22 molecule	chr19q13.1	0,44	1,43E-86	BCR signaling
PLEKHF2	pleckstrin homology domain containing, family F (with FYVE domain) member 2	chr8q22.1	0,43	5,33E-80	BCR signaling
RRAS2	related RAS viral (r-ras) oncogene homolog 2	chr11p15.2	0,42	2,95E-77	BCR signaling
LPXN	leupaxin	chr11q12.1	0,41	1,60E-75	BCR signaling
LRMP	lymphoid-restricted membrane protein	chr12p12.1	0,40	3,07E-71	BCR signaling

Supplemental Table 5. Prioritized drug-gene targets. List of drug-gene interactions for the first 500 MS4A1-associated gene probes, with corresponding pubmed IDs.

Search Term	Gene	Drug	PubMed IDs
ACTR2	ACTR2 - ARP2 actin-related protein 2 homolog (yeast)	CK-636	10592235
ACTR3	ACTR3 - ARP3 actin-related protein 3 homolog (yeast)	CK-636	10592235
ALOX5AP	ALOX5AP - arachidonate 5-lipoxygenase-activating protein	VELIFLAPON, AM103	
ARPC2	ARPC2 - actin related protein 2/3 complex, subunit 2, 34kDa	CK-636	10592235
ARPC3	ARPC3 - actin related protein 2/3 complex, subunit 3, 21kDa	CK-636	10592235
ARPC4	ARPC4 - actin related protein 2/3 complex, subunit 4, 20kDa	CK-636	10592235
ARPC5	ARPC5 - actin related protein 2/3 complex, subunit 5, 16kDa	(2S)-2-(3-BROMOPHENYL)-3-(5-CHLORO-2-HYDROXYPHE...	10592235
BIRC3	BIRC3 - baculoviral IAP repeat containing 3	CK-636	
BLK	BLK - B lymphoid tyrosine kinase	ENMD-2076	
BTK	BTK - Bruton agammaglobulinemia tyrosine kinase	IBRUTINIB, AVL-292	24869598

Supplemental Table 5 continued.

Search Term	Gene	Drug	PubMed IDs
CD19	CD19 - CD19 molecule	SAR3419	
BTK	BTK - Bruton agammaglobulinemia tyrosine kinase	IBRUTINIB, AVL-292	24869598
CD19	CD19 - CD19 molecule	SAR3419	
CDK1	CDK1 - cyclin-dependent kinase 1	DINACICLIB	
CNR2	CNR2 - cannabinoid receptor 2 (macrophage)	NABILONE, DRONABINOL	
CREB1	CREB1 - cAMP responsive element binding protein 1	NALOXONE	12969258, 14653953, 12956944, 15953421, 16417577
DCK	DCK - deoxycytidine kinase	FLUDARABINE, DEOXYCITIDINE	
DHFR	DHFR - dihydrofolate reductase	METHOTREXATE, PRALATREXATE	
ESR2	ESR2 - estrogen receptor 2 (ER beta)	TAMOXIFEN	17404078, 16326830, 16822624, 2011412, 11752352, 17158759, 20827267, 16010412
FDFT1	FDFT1 - farnesyl-diphosphate farnesyltransferase 1	NB-598 , BPH-830	
FNTA	FNTA - farnesyltransferase, CAAX box, alpha	TIPIFARNIB, AZD3409	
GATM	GATM - glycine amidinotransferase (L-arginine:glycine amidinotransferase)	DELTA-AMINO VALERIC ACID	17486546, 16970975
GMDS	GMDS - GDP-mannose 4,6-dehydratase	DB02547	17016423, 17139284, 10592235
GPR18	GPR18 - G protein-coupled receptor 18	CANNABIDIOL	
HDAC1	HDAC1 - histone deacetylase 1	PANOBINOSTAT, VALPROIC ACID, VORINOSTAT, ROMIDEPSIN	
HTR3A	HTR3A - 5-hydroxytryptamine (serotonin) receptor 3A, ionotropic	GRANISETRON, MIRTAZAPINE	
ITPR1	ITPR1 - inositol 1,4,5-trisphosphate receptor, type 1	CAFFEINE	1844813
LYN	LYN - v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	BOSUTINIB, PONATINIB	
MAP3K7	MAP3K7 - mitogen-activated protein kinase kinase kinase 7	RGB-286638	
MAPK1	MAPK1 - mitogen-activated protein kinase 1	ERLOTINIB	26181029
MDH1	MDH1 - malate dehydrogenase 1, NAD (soluble)	2'-MONOPHOSPHOADENOSINE 5'-DIPHOSPHORIBOSE	10592235
METAP2	METAP2 - methionyl aminopeptidase 2	BELORANIB	
NPM1	NPM1 - nucleophosmin (nucleolar phosphoprotein B23, numatrin)	NSC348884	21719597
PAPOLA	PAPOLA - poly(A) polymerase alpha	3-SULFINOALANINE	17016423, 17139284, 10592235

Supplemental Table 5 continued.

Search Term	Gene	Drug	PubMed IDs
PARP1	PARP1 - poly (ADP-ribose) polymerase 1	OLAPARIB, VELIPARIB	
PKD3	PKD3 - pyruvate dehydrogenase kinase, isozyme 3	CPI-613	
PLCG2	PLCG2 - phospholipase C, gamma 2 (phosphatidylinositol-specific)	IBRUTINIB	24869598
PPP1CA	PPP1CA - protein phosphatase 1, catalytic subunit, alpha isozyme	SCY-635 Vitamin E	
PRKAB1	PRKAB1 - protein kinase, AMP-activated, beta 1 non-catalytic subunit	METFORMIN	
PRKD3	PRKD3 - protein kinase D3	QUERCETIN	
PSMD6	PSMD6 - proteasome (prosome, macropain) 26S subunit, non-ATPase, 6	CARFILZOMIB	
PSME3	PSME3 - proteasome (prosome, macropain) activator subunit 3 (PA28 gamma; Ki)	BORTEZOMIB	
QPCT	QPCT - glutaminyl-peptide cyclotransferase	N-ACETYLBISTAMINE	
RAB8B	RAB8B - RAB8B, member RAS oncogene family	DB02082	17016423, 17139284, 10592235
ROCK1	ROCK1 - Rho-associated, coiled-coil containing protein kinase 1	HYDROXYFASUDIL	11752352, 10592235
RPL19	RPL19 - ribosomal protein L19	PUROMYCIN	10592235
RRM1	RRM1 - ribonucleotide reductase M1	CLADRIBINE, HYDROXYUREA	16316309, 9923554, 19576186, 19715446, 17852710
SDHC	SDHC - succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa	SUCCINIC ACID	15986240, 17636259, 16405730, 16288654, 16232921
SYK	SYK - spleen tyrosine kinase	PACLITAXEL, FOSTAMATINIB	26096845
UGCG	UGCG - UDP-glucose ceramide glucosyltransferase	MIGLUSTAT	
WEE1	WEE1 - WEE1 homolog (S. pombe)	MK-1775	
XRCC4	XRCC4 - X-ray repair complementing defective repair in Chinese hamster cells 4	DOCETAXEL

Supplemental Figure 1. Overall survival for patients with diffuse large B-cell lymphoma (DLBCL). (A), Germinal Center B-Cell (GCB) DLBCL (B), Activated B-cell (ABC) DLBCL (C), and unclassified DLBCL (D) treated with CHOP or R-CHOP. Log-rank testing was used to test whether the curves are statistically different (* p -value < 0.0001 , ** p -value 0.003). Abbreviation: R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone.

Supplemental Figure 2. In vitro activity of rituximab, AZD1775 and olaparib. (A) CD20 expression level of 8 different Diffuse Large B-cell Lymphoma (DLBCL) cell lines. The cell-of-origin is indicated of each individual cell line. (B) The in vitro susceptibility of the DLBCL cell line to rituximab in the presence of human complement. (C) Western blot results of WEE1, PARP1 and γ H2AX protein expression of SUDHL6 and SUDHL10 treated for 24 hours with 1 μ M AZD1775 or 250 μ M Olaparib. (D) Resazurin metabolic activity assay of the WEE1 inhibitor AZD1775 with or without rituximab in two rituximab sensitive and two resistant cell lines: SUDHL6, SUDHL10, U2932, and SC-1. Shown is the normalized metabolic activity of three independent analyses. Data was analyzed with student T-test as compared to sample without inhibitor treatment. Significant (*) $p \leq 0.01$ / (**) $p \leq 0.001$ / (***) $p \leq 0.0001$.

WEE1 inhibition synergizes with CHOP chemotherapy and radiation therapy through induction of premature mitotic entry and DNA damage in Diffuse Large B-cell Lymphoma

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Abstract

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.

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.

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 to CHOP or RT alone. Of the single CHOP components, doxorubicin showed the strongest effect together with AZD1775, reducing viability and increasing DNA damage.

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 and/or DNA damaging (chemo)therapeutic compounds and should be further explored in clinical trials.

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% 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 rituximab in DLBCL [4,5]. Novel therapies that combine R-CHOP with bortezomib [6], lenalidomide [7] or ibrutinib [8] 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 (AID) induces oncogenic mutations and chromosomal translocations [12], causing genetic instability [13] 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 and/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% fetal 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 STR 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 ¹³⁷Cesium source-662 keV photons (IBL 637, Cis Bio International, Gif-sur-Yvette, France) and metabolic activity of cells was measured after 72 hours. 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 hours treatment of 0.4 x 10⁶ cells/ml. Cells were incubated with 10 µl resazurin (5% final concentration, AlamarBlue, Thermo Fisher Scientific, Waltham, MA USA) for nine hours prior to read-out (Varioskan, excitation 560nm, emission 590nm). Experiments were performed 5 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% BSA/PBS and resuspended in solution containing 0.1% sodium citrate (A0158348, Merck, Kenilworth, New Jersey, United States), 0.01% propidium iodide (P4170, Sigma-Aldrich, St. Louis, Missouri, United States), 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 3 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 analyzed using Kaluza 1.5 analysis software (Beckman). Experiments were performed 3 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 analyzed 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 3 times.

Western blot

Cells were washed with PBS and lysed in RIPA buffer (50mM Tris, 150mM NaCl, 2.5mM Na2EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS in dH2O) with 1mM phenylmethanesulphonyl fluoride for 30-45 minutes on ice. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (#23227; Thermo Scientific, Waltham MA, USA). Samples were loaded at 40µg 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: $1 (\text{factor of AZD alone}) * \text{IC50 AZD+treatment} \div \text{IC50 AZD alone}$. If the IC50 value could not be calculated the value is marked with “not available” (n.a.). Synergism of combination therapies was calculated using the method of Combination Subthresholding, which calculates as the difference between the expected effect (E) of the combination therapy compared to 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 $p \leq 0.05$, ** indicates $p \leq 0.01$ and *** indicates $p \leq 0.001$.

Results

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

Radiation therapy (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 IC50 value of 357 nM for OCI-LY3 to a maximum IC50 value of 1835 nM for SUDHL-4, values that are comparable to or less than the clinical Cmax serum concentration for AZD1775 of 1650 nmol/L in solid tumours [17] (Supplemental figure 1A). The combination of AZD1775 with RT showed a dose-dependent decrease in the IC50 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 figure 2A). Combination of AZD1775 with 4 Gy radiation resulted in a 5.5-fold increased sensitivity to AZD1775 (IC50 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 IC50 values of all cell lines, resulting in synergism in seven out of eight cell lines (Figure 1B and Supplemental figure 2B). An up to 6-fold enhanced sensitivity to AZD1775 was observed in SUDHL-10 (IC50 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 IC50 values for AZD1775 ($p=0.2500$) (Supplemental figure 1B), CHOP ($p=0.5446$) (Supplemental figure 1C) or RT ($p=0.1876$) (Supplemental figure 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.

Figure 1. AZD1775 combination therapy with either 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 Gy (red) or 4 Gy (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). 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. 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), 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" (n.a.). 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, analyzed for significance using a one-sample T-test. Colours represent additive in white (E=O), synergy in green (E<O) and antagonism in red (E>O).

AZD1775 with RT or CHOP therapy activates the DNA damage response

Next, we investigated whether 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.

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 AZD1776 combined with 2 Gy or 4 Gy 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 AZD1776 combined with 0.1 or 1 μ g/mL CHOP. Data are plotted as the mean $\pm$ S.D. of $n=3$. Samples of untreated control cells were compared to treated cells, unless otherwise marked with capped lines. Statistical analysis was performed using a one-sample paired T-test (* $p \leq 0.05$) (** $p \leq 0.01$) (***) $p \leq 0.001$).

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% to 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].

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) SUDHL-5, (B) SUDHL-6 and (C) SUDHL-10 cell lines treated with 500 nM AZD1775, 4 Gy RT and 1 μ g/mL CHOP for 3 hours. Inhibition of CDC2 is facilitated by phosphorylation on Tyr15 by WEE1, and therefore a marker for WEE1 activity. Induction of DNA damage results in downstream activation/phosphorylation of Ser345 on CHK1 (single-stranded DNA breaks) and/or Thr68 on CHK2 (double-stranded DNA breaks). Increased expression of phosphorylated Ser10 on histone H3 was measured as a marker for G2/M cell cycle arrest. GAPDH was measured as a loading control.

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 hours increased phospho-CHK1 levels (Ser345). These effects were more prominent when RT was combined with AZD1775. While 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-CBK2 (Thr68) were increased (Figure 3A) upon treatment with RT and CHOP monotherapy, however no further increase was observed by addition of AZD1775. In the cell lines SUDHL-6 (Figure 3B) treatment with RT strongly induced phospho-CBK2, 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-CBK2 induction was observed upon RT or CHOP treatment, whereas addition of AZD1775 to RT strongly enhanced phospho-CBK2 expression.

In all three cell lines mentioned above, treatment with AZD1775 led to a significant reduction in phospho-CDC2 (Tyr15) 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 CBK1 and CBK2 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-CBK1 and phospho-CBK2) 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 (a marker for premature mitosis) 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 to 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 μ g/mL, whereas CHOP treatment at 1 μ g/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 previously observed for the combination of AZD1775 with RT (Figure 4A).

Figure 4. Cell cycle and p_{H3} 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 Gy or 4 Gy 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 Gy or 4 Gy 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 $\pm$ S.D. of $n=3$. Samples of untreated control cells were compared to treated cells, unless otherwise marked with capped lines. Statistical analysis was performed using a one-sample paired T-test ($p \leq 0.05$) ($**p \leq 0.01$) ($***p \leq 0.001$).

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 µg/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 hours (Figure 5A-B). Protein analysis showed that RT led to an increased phospho-CDC2 level, indicating increased WEE1 activity, which was subsequently reduced to control levels on addition of AZD1775 (Figure 5A). This 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 phospho-CDC2 levels were observed upon CHOP monotherapy treatment (Figure 5BA). 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.

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 (Ser139; double-stranded DNA breaks) and GAPDH protein levels in SUDHL-10 treated with 0.5 μ M AZD1775 in combination with 4 Gy 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.

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 Cmax dosages [19]. Cyclophosphamide monotherapy at low non-toxic dosages increased WEE1, phospho-CDC2 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 phospho-CDC2 and γ H2AX levels compared to control cells, which was absent in cells treated with a toxic concentration of 10 μ M (Figure 5C). Unlike cyclophosphamide and doxorubicin, vincristine monotherapy actually reduced both phospho-CDC2 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 whether 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 to 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.

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 ± S.D. of $n=3$. Samples of untreated control cells were compared to treated cells, unless otherwise marked with capped lines. Statistical analysis was performed with a one-sample paired T-test (* $p \leq 0.05$) (** $p \leq 0.01$) (** $p \leq 0.001$) ($n=3$).

Analysis of γH2AX showed that doxorubicin monotherapy induced minimal γH2AX positive cells, but addition of AZD1775 significantly increased γH2AX from 2% to 13% ($p=0.0083$) (Figure 6E). Vincristine was particularly effective in inducing DNA damage, especially in combination with AZD1775 (γH2AX 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 to 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% to 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 instability [13] 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 tumours 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 important [21]. 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 cancer [17] and ovarian adenocarcinoma tumour cells [15]. However, other studies in 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 lines [24] 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 radiation therapy has proven successful in gliomas and osteosarcoma [30,31]. Recently, Wang et al. 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 [24,26]. 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 hours after treatment, but both WEE1 and pCDC2 levels declined at 24 hours. By contrast, pCDC2 levels steadily increased in TP53 mutant cells after treatment, even at 24 hours [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 1) G2/M arrest, 2) WEE1 upregulation, 3) activation of the DDR, or 4) 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 et al. showed that at the spindle assembly checkpoint (SAC) 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 et al. 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.

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Supporting information

Supplemental Figure 1. TP53 mutation status of DLBCL cell lines. (A) Overview of cell or origin (COO), TP53 mutation status and IC50 for AZD1775, CHOP and RT in eight DLBCL cell lines. Comparison of IC50 of TP53 wild type (WT) or mutated (MUT) DLBCL cell lines for (B) AZD1775, (C) CHOP and (D) radiation therapy.

Supplemental Figure 2. Combination therapy of AZD1775 with radiation therapy radiation (RT) and CHOP. (A) Raw data for eight DLBCL cell lines treated for 72 hours with 100 nM or 500 nM AZD1775 combined with 2 Gy or 4 Gy radiation. (B) Raw data for eight DLBCL cell lines treated for 72 hours with 100 nM or 500 nM AZD1775 combined with 0.01 μ g/mL, 0.1 μ g/mL or 1 μ g/mL CHOP. Data were normalized to the control and plotted as the mean $\pm$ S.D. of $n=3$. Statistical analysis was performed using a one-sample paired T-test (* $p \leq 0.05$) (** $p \leq 0.01$) (***) $p \leq 0.001$) ($n=3$).

4

WEE1 inhibition improves effectivity of cytarabine, but not cisplatin therapy in diffuse large B-cell lymphoma

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Abstract

First line treatment for diffuse large B cell lymphoma (DLBCL) consist of cyclophosphamide, vincristine and doxorubicin together with the immunosuppressive agent prednisone and the anti-CD20 antibody rituximab (R-CHOP) and cures roughly 60% of DLBCL patients. In relapsed or refractory patients, treatment consists of chemotherapeutic combinations, often including anti-metabolite cytarabine or the platinum-based alkylating agent cisplatin. We recently identified cell cycle regulator WEE1 as a strong candidate for targeted-treatment in DLBCL, and demonstrated favorable combinations with first line therapies rituximab, CHOP and radiation.

In the current study, we investigated the synergistic effect of the WEE1 inhibitor AZD1775 when combined with chemotherapeutic compounds cytarabine and cisplatin in DLBCL cell lines. Based on cell viability data, cell cycle analysis and DNA damage analysis we confirmed strong synergism for AZD1775 and cytarabine, which enhanced DNA damage levels and cell death. In contrast, AZD1775 combined with cisplatin induced very little additional cell death in DLBCL cell lines, which was corroborated by a lack of additional DNA damage. Further analysis revealed a decrease of WEE1 protein and mRNA levels upon cisplatin monotherapy treatment, explaining the lack of synergism, while no effect was observed after cytarabine or AZD1775 treatment. Moreover, chromatin immunoprecipitation (ChIP) showed a decrease in active transcription marks H2K4Me3 and H3K27Ac, indicating reduced WEE1 gene transcription as a results of cisplatin treatment.

These results demonstrate that although DNA damaging agents might seem a rational choice for AZD1775 combination therapy, not all genotoxic agents do synergize with WEE1 inhibition and it is important to first evaluate its synergistic or any potential antagonistic effects.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin lymphoma (NHL) subtype for which standard therapy of rituximab with cyclophosphamide, vincristine, doxorubicin and prednisone (R-CHOP) cures approximately 60% of patients. For patients with relapse or refractory disease, different treatment options are available, which often include cytarabine [1] and/or cisplatin [2]. However, 40% of patients that undergo second line therapy still develop refractory disease. To improve survival especially for this group, additional targets are needed to improve the treatment outcome.

We recently identified the cell cycle regulator WEE1 as a promising novel target in DLBCL cell lines either as a single agent or in combination with rituximab [3]. Targeting of WEE1 with the inhibitor AZD1775 was shown to induce both premature mitotic entry [4,5] and DNA damage [6], making it a promising candidate to test alongside genotoxic drugs. Indeed, combination of AZD1775 and cytarabine was shown to be synergistic in TP53-deficient and wild type AML cell lines [7] and in B-cell lymphoma cells lines [8]. Moreover, an RNAi screen in AML cell lines identified WEE1 inhibition as the most potent sensitizer with cytarabine, outperforming both ATR and CHK1 [9]. Similar results have been achieved for the combination of cisplatin with WEE1 inhibitors in medulloblastoma cell lines [10]. Furthermore, WEE1 inhibition was able to overcome cisplatin resistance in triple negative breast cancer cell lines [11] and head and neck cancer cell lines [12].

In the current study, we investigated the synergistic effect of AZD1775 combined with second line chemotherapeutic compounds cytarabine and cisplatin in DLBCL cell lines. Based on cell viability, cell cycle and DNA damage analysis we showed that WEE1 inhibition is synergistic with cytarabine but not cisplatin chemotherapy. The lack of synergism for cisplatin was caused by the cisplatin-induced reduction of WEE1 protein expression as a result of reduced WEE1 transcription. These data show that not all genotoxic agents synergize with WEE1 inhibition. This implicates that although DNA damaging agents like cytarabine or cisplatin might seem like obvious choices for AZD1775 combination therapy, careful evaluation of such combination therapies must be performed before implementation into the clinic.

Materials and methods

Cell lines and culture conditions

DLBCL cell lines U-2932, SUDHL-2 and SC-1 were cultured in suspension in Roswell Park Memorial Institute medium 1640 (RPMI 1640; Lonza BioWhittaker, Walkersville, MD, USA) with 10% Fetal Bovine Serum (FBS; HyClone Thermo Scientific, Waltham, MA, USA), 1%

Penicillin-Streptomycin (PS; Lonza BioWhittaker) and 1% Glutamine (Lonza BioWhittaker). DLBCL cell line 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 checked at a regular base.

Compounds

WEE1 inhibitor AZD1775 was acquired from Selleckchem (No.S1525, Houston, TX, USA). Incubations with AZD1775 were done at sub-lethal concentrations of 100 nM and 500 nM. Cytarabine (Ara-C) was acquired from Selleckchem (No.S1648) and incubated for 96 hours. Cisplatin was acquired from UMCG pharmacy (Accord Healthcare Limited) and incubated for 72 hours.

Flow cytometry – cell cycle and γ H2AX

For cell cycle analysis, 0.2×10^6 cells/ml were treated for the appropriate time points, washed with 1% BSA/PBS and resuspended in propidium iodide solution containing 0.1% sodium citrate (A0158348, Merck, Kenilworth, New Jersey, United States), 0.01% propidium iodide (P4170, Sigma-Aldrich, St. Louis, Missouri, United States), 0.002% RNase A (R4875, Sigma-Aldrich) and 0.3% Triton X100 (T9284, Sigma-Aldrich). Samples were processed on BD FACSCalibur 2 and analysed with ModFit LT (Verity Software House). Experiments were performed 3 times.

For γ H2AX analysis, cells at 0.2×10^6 cells/ml were treated for the appropriate time points, and stained with mouse-anti- γ H2AX-AlexaFluor-647 (2F3: 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 MACSQuant and data was analysed with Kaluza 1.5 analysis software (Beckman). Experiments were performed 3 times.

Western blot

Cells were washed with PBS and lysed in RIPA buffer (50mM Tris / 150mM NaCl / 2.5mM Na₂EDTA / 1% Triton X-100, 0.5% sodium deoxycholate/0.1% SDS in dH₂O) with 1mM phenylmethanesulphonyl fluoride for 30-45 minutes on ice. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (#23227; Thermo Scientific, Waltham MA, USA). Samples were loaded at 40µg 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 Milipore, Temecula, CA,

USA) and GAPDH (1:10,000; NovusBio) was done overnight and staining at 4°C.

RT-qPCR

cDNA was synthesized using random primers, dNTP mix and the Superscript II Reverse Transcriptase Kit (Life Technologies Europe BV, Bleiswijk, the Netherlands) according to the manufacturers standard protocol. A total input of 500 ng RNA was used per sample in a reaction volume of 20 µL. SYBRgreen mix (Applied Biosystems) was used to detect transcript levels in a qPCR reaction volume of 10 µL containing 1 ng cDNA on a Lightcycler 480 (Roche, Penzberg, Germany). WEE1 primer forward sequence: 5'-GATGTGCGACAGACTCCTCAAG-3'; reverse sequence 5'-CTGGCTTCCATGTCTTCACCAC-3'. WEE1 TaqMan gene expression assay HS01119384_G1 (ThermoFisher, Waltham MA, USA).

Chromatin immunoprecipitation (ChIP)

For ChIP analysis, cells were collected and cross-linked using 1% formaldehyde (Sigma-Aldrich) and sonicated using a Diagenode Bioruptor. Sheared chromatin fragments were incubated with specific antibodies against histone marks H3K4me3 (Diagenode C15410003-50; RRID:AB_2616052), H3K27ac (Diagenode C15410196; RRID:AB_2637079), H3K27me3 (Diagenode C15410195; RRID:AB_2753161) and H2AK119ub (D27C4, Cell Signaling Technology). After immunoprecipitation, cross-linking was reversed and the DNA used for quantitative PCR and sequencing analysis. As a control, a portion of the chromatin was processed without the immunoprecipitation step. Each sample was treated with the Kapa Hyper Prep Kit to create an Illumina library and subjected to sequencing on the Illumina HiSeq 2000 machine.

Statistical analysis

Data was analyzed using Graphpad PRISM (version 5.0) software and tested for significant differences with a paired T-test. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$ and *** indicates $p \leq 0.001$. Synergism was determined by comparing the observed effect of a treatment compared to the expected effect of a treatment as based on the single treatment outcome. Differences between expected and observed effects were tested with a paired T-test. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$ and *** indicates $p \leq 0.001$.

Results

AZD1775 synergizes with cytarabine therapy in DLBCL cell lines

To establish whether WEE1 inhibition would synergize with cytarabine, we tested the combination treatment at suboptimal concentrations for each compound (Supplemental table 1) in four DLBCL cell lines and observed that the combination significantly reduced cell viability in all cell lines (Figure 1A and Supplemental figure 1A). Cell viability decreased from 61% to 28% ($p=0.0205$) in SUDHL-10, from 78% to 37% ($p=0.0164$) in SC-1, from 74% to 39% ($p=0.0200$) in U-2932 and from 61% to 28% ($p=0.0205$) in SUDHL-2. This combination was synergistic in SUDHL-10 ($\Delta 46\%$ $p=0.0007$), additive in SC-1 ($\Delta 14\%$ $p=0.1008$), synergistic in U-2332 ($\Delta 40\%$ $p=0.0397$) and additive in SUDHL-2 ($\Delta 3\%$ $p=0.7365$) (Supplemental table 2). No correlation was found between TP53 mutation status of our cell lines and the levels of synergism for the cytarabine and AZD1775 combination treatment.

Figure 1. Combination therapy of cytarabine or cisplatin with AZD1775. (A) Cell viability data of SUDHL-10 and SC-1 treated for 96 hours with cytarabine and AZD1775. Data was normalized to the control and plotted as the mean ± S.D. of n=3. (B) Cell cycle analysis of changes in S-phase in SUDHL-10 and SC-1 treated with cytarabine and AZD1775 for 24 hours (n=3). (C) yH2AX analysis of SUDHL-10 and SC-1 treated with cytarabine and AZD1775 for 24 hours (n=3).

(D) Cell viability data of SUDHL-10 and SC-1 treated for 72 hours with cisplatin and AZD1775. Data was normalized to the control and plotted as the mean $\pm$ S.D. of $n=3$. (E) Cell cycle analysis of changes in S-phase in SUDHL-10 and SC-1 treated with cisplatin and AZD1775 for 24 hours ($n=3$). (F) γ H2AX analysis of SUDHL-10 and SC-1 treated with cisplatin and AZD1775 for 24 hours ($n=3$). Statistical analysis was performed with paired T-test. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$ and *** indicates $p \leq 0.001$.

Analysis of the cell cycle distribution in cell lines SUDHL-10 and SC-1 showed a small dose-dependent increase of the percentage of S-phase cells in SUDHL-10 cells treated with cytarabine, which was further increased by addition of AZD1775 (Figure 1B and Supplemental figure 2A). No changes were observed in cell line SC-1. Analysis of DNA damage marker γ H2AX showed weak induction of DNA damage by AZD1775 or cytarabine alone, but a strong increase in the combination therapy (Figure 1C and Supplemental figure 2B). In SUDHL-10, DNA damage levels increased from 2% to 36% ($p=0.0023$) and in SC-1 from 20% to 72% ($p=0.0007$). These results strongly support the potential benefit for a combination therapy of AZD1775 with cytarabine, which acts synergistically to reduce cell viability and increase DNA damage levels.

AZD1775 does not synergize with cisplatin therapy in DLBCL cell lines

Next, we tested the combination of alkylating agent cisplatin with AZD1775. In contrast to cytarabine, addition of AZD1775 did not outperform the single cisplatin treatment (Figure 1D and Supplemental figure 1B). For SUDHL-10, cell viability levels decreased from 50% to 42% ($p=0.4443$), but this reduction was not significant. For SC-1 cell viability increased from 47% to 61% ($p=0.0782$), whereas no effect on cell viability was observed in U-2932 cells. In the highly cisplatin-sensitive cell line SUDHL-2, a significantly decreased viability was observed going from 88% to 25% ($p=0.0085$) (Supplemental figure 1B). Overall, addition of AZD1775 with cisplatin treatment showed no significant synergism, but only additive or antagonistic effects (Supplemental table 2). No correlation was found between TP53 mutation status or the level of antagonism/synergism for the cisplatin and AZD1775 combination treatment.

Analysis of the cell cycle distribution showed a significantly increased percentage of S-phase cells in SUDHL-10 but not in cell line SC-1 upon cisplatin treatment (Figure 1E and Supplemental figure 3A). Addition of AZD1775 reduced S-phase levels from 62% to 46% ($p=0.0652$) in SUDHL-10, whereas in SC-1 addition of AZD1775 enhanced S-phase cells at 10 μ M Cisplatin and had no effect at 50 μ M on the cell cycle distribution. Analysis of DNA damage levels showed significantly increased γ H2AX levels in SUDHL-10 (from 11% to 43%; $p=0.0351$) (Figure 1F and Supplemental figure 3B), which however did not result in any significant additional cell death (Figure 1D). In SC-1, addition of AZD1775 DNA damage levels increased from 30% to 68% ($p=0.0017$) when combined with 10 μ M cisplatin and from 66% to 68% ($p=0.0870$) when combined with 50 μ M cisplatin, indicating that cisplatin treatment alone already induced the maximum levels of DNA damage. These results are consistent with the cell viability data for SC-1, which showed a small additive effect for AZD1775 addition at a lower cisplatin concentration of 10 μ M, which turns into antagonism at a higher cisplatin concentration of 50 μ M. Taken together, these results suggest that

cisplatin-sensitive cells which are treated with a lower cisplatin dosage might still benefit from additional AZD1775 treatment, whereas cisplatin-insensitive cells which will require a higher cisplatin dosage will most likely not benefit from additional AZD1775 treatment.

Cisplatin treatment decreases WEE1 protein and mRNA levels

To elucidate why cytarabine but not cisplatin synergizes with AZD1775, we performed western blot analyses for WEE1, its downstream target phosphorylated CDC2 and the DNA damage marker γ H2AX in SUDHL-10 and SC-1 (Figure 2A). In addition, we performed a WEE1 RT-qPCR analysis (Figure 2B).

Figure 2. WEE1 protein and gene expression analysis after cytarabine or cisplatin and AZD1775 therapy. (A) Representative western blot WEE1, pCDC2 and γ H2AX protein levels in SUDHL-10 and SC-1 treated with cytarabine, cisplatin and AZD1775 or combined for 24 hours. All samples were run on the same gel. **(B)** mRNA expression of WEE1 in SUDHL-10 and SC-1 treated with cytarabine, cisplatin and AZD1775 or combined for 24 hours.

Both cell lines showed a decrease in pCDC2 protein levels as a result of AZD1775 treatment, indicating reduced WEE1 activity, whilst no or only mild reduction of WEE1 protein levels were observed. At the same time, γ H2AX levels were increased, indicating WEE1 inhibition induced DNA damage. Cytarabine treatment induced WEE1 and pCDC2 protein expression in SUDHL-10, but not in SC-1, suggesting cell cycle arrest was activated to cope with the DNA damage induction, as confirmed by the increase in γ H2AX protein levels. The combination of cytarabine and AZD1775 increased γ H2AX levels further, indicating combination treatment was more effective compared monotherapies of cytarabine or AZD1775. For cisplatin, monotherapy caused increased protein expression of pCDC2 and γ H2AX in SUDHL-10, indicating cell cycle arrest and DNA damage. In contrast, cisplatin monotherapy in SC-1 caused significant reduction of WEE1 and pCDC2 protein levels, despite induction of DNA damage marker γ H2AX. These effects were further enhanced in the combination therapy, leaving WEE1 and pCDC2 protein levels nearly undetectable. This phenomenon was not observed in cell line SUDHL-10, which was treated with a lower

cisplatin concentration, indicating this could be either a dose-dependent of cell line specific observation. At the RNA level, WEE1 expression was not affected by treatment with AZD1775, cytarabine, cisplatin or the combinations in SUDHL-10. In SC-1, WEE1 transcript levels were strongly induced by treatment with AZD1775 monotherapy and mildly reduced by cisplatin monotherapy or in combination with AZD1775 in SC-1 (Figure 2B). The slightly reduced WEE1 transcript levels are consistent with the reduced WEE1 protein levels. These results give a possible indication for the lack of synergism for cisplatin and AZD1775, as WEE1 protein expression are strongly reduced in response to cisplatin treatment.

Figure 3. WEE1 protein and gene expression dosage effect of cisplatin treatment. (A) Representative western blot of WEE1, pCDC2 and γH2AX protein levels in SUDHL-10 and SC-1 treated with increasing concentration of cisplatin for 24 hours. (B) mRNA expression of WEE1 in SUDHL-10 and SC-1 treated with increasing concentration cisplatin for 24 hours.

Based on previous results which showed strong cisplatin-induced reduction of WEE1 protein expression, we next tested at which cisplatin concentration we could start to observe WEE1 protein reduction. As a control, we also tested a dilution range of cytarabine treatment. (Figure 3A-B and Supplemental figure 6). Western blot analysis cisplatin-treated SUDHL-10 cells showed a dose-dependent increase in pCDC2 and γH2AX protein levels, with only minor changes in WEE1 protein levels (Figure 3A). This suggests that cisplatin increases WEE1 activity in a dose-dependent manner, which is likely part of a DNA damage response (DDR) pathway activation in response to the induction of DNA damage. In SC-1 a marked reduction of both WEE1 and pCDC2 protein levels were observed, starting at a dosage of 50 μM cisplatin. At the same concentration, marked increase in protein levels of γH2AX were observed (Figure 3A). These data indicate that although cisplatin is able to induce significant levels of DNA damage in SC-1, these changes are accompanied by a reduction in WEE1 activity, making combination therapies with WEE1 inhibitors ineffective. These results are in line with WEE1 gene expression analysis, which showed a reduction in WEE1 mRNA levels in cell line SC-1, but not in SUDHL-10 (Figure 3B).

Since SUDHL-10 showed higher sensitivity to cisplatin, combination treatment was performed at a much lower dosage of cisplatin, at which no adverse effects on WEE1 protein or mRNA expression are observed. As expected, treatment with cytarabine showed

no changes in WEE1 and pCDC2 protein levels, but a steady dose-dependent increase of γ H2AX protein levels, indicating cytarabine induces DNA damage in a dose-dependent manner without influencing WEE1 activity (Supplemental figure 4A). Similarly, qPCR analysis revealed no or only minor alterations in at the WEE1 transcript level after cytarabine treatment (Supplemental figure 4B). Taken together, these results indicate that in cisplatin-sensitive cells, cisplatin could work synergistic alongside AZD1775, however the combination could be antagonistic in cisplatin-insensitive cells due to a reduction in WEE1 expression.

Cisplatin treatment decreases WEE1 gene transcription

Based on the above described results showing that treatment with cisplatin results in regulate decrease of WEE1 at the protein and mRNA expression levels, we next analyzed treatment induced changes in histone marks by performing chromatin immunoprecipitation (ChIP) for active histone marks H2K4 trimethylation (H2K4Me3) and H3K27 mono-acetylation (H3K27Ac) and repressive histone marks H2AK119 mono-ubiquitination (H2AK119Ub) and H3K27 trimethylation (H3K27Me3) (Figure 4).

Figure 4. Chromatin immunoprecipitation of WEE1 in cell line SC-1. Cell line SC-1 was treated with 100 nM cytarabine or 100 μ M cisplatin after which chromatin immunoprecipitation was performed of the WEE1 gene for active marks H2K4 trimethylation (H2K4Me3) and H3K27 mono-acetylation (H3K27Ac) and repressive marks H2AK119 mono-ubiquitination (H2AK119Ub) and H3K27 trimethylation (H3K27Me3). Results are plotted as relative enrichment of 10% input and plotted as the mean $\pm$ S.D. of $n=3$. X-axis represent the position relative to the WEE1 gene with zero being the transcription start site (TSS).

Analysis of ChIP data for cytarabine treated cells showed treatment had no effect on

both active and repressive histone marks of the WEE1 gene, which is in line with the evidence that cytarabine treatment does not influence WEE1 protein or mRNA levels. In contrast, a strong decrease in active histone marks at the transcription start site of the WEE1 gene was observed upon treatment with cisplatin as compared to untreated cells, with a decrease of 30% for the H2K4Me3 histone mark and with 2% for the H3K27Ac histone mark. This suggests that there is less active transcription taking place of the WEE1 gene as a result of cisplatin treatment, consistent with the decrease in both WEE1 protein and mRNA levels. No changes were observed in presence of the repressive H2AK119Ub and H3K27Me3 histone marks, indicating there is no active repression of WEE1 gene transcription as a result of cisplatin treatment. These results show that the reduced WEE1 protein expression induced by cisplatin is the results of a decrease in active WEE1 gene transcription, and not the result of active gene repression. Taken together, these data show that cisplatin treatment will not only induce DNA damage as a result of DNA intercalation, but might thereby also alter WEE1 gene transcription as a secondary effect, which could have an adverse outcome in WEE1 inhibitor-based combination therapies.

Discussion

In the current study, we examined the potential combination of second line chemotherapeutic agents cytarabine and cisplatin with WEE1 inhibitor AZD1775 in DLBCL. For the anti-metabolite cytarabine, we found synergistic results when combined with AZD1775, which was caused by a significant increase in DNA damage levels in the combination therapy. The DNA damaging effect of cytarabine is acquired through its incorporation into the DNA as the active triphosphate form, preventing general DNA replication and repair, but also inhibiting DNA and RNA polymerases [13]. Like most genotoxic agents, cytarabine treatment leads to CHK1 activation [14], which is required for WEE1 activation upon DNA damage [15], making it both in theory and in practice a suitable agent for combination with WEE1 inhibition. Wang et al. previously observed similar synergistic results for WEE1 inhibition in B-cell lymphoma cell lines arrested in the G2/M phase by cytarabine treatment, but not in cell lines arrested in the G1/S phase, which they hypothesized was the determining factor for synergism between WEE1 inhibition and cytarabine [8]. In our data, we observed little effect of cytarabine on the cell cycle, which could be explained by the use of a lower cytarabine dosage compared to Wang SD. However, despite the lack of a strong cell cycle arrest we still observed synergistic cell death for the combination therapy, suggesting cell cycle arrest may not be the determining factor for synergism, as long as significant levels of DNA damage are induced in the combination therapy.

Unlike the success story for cytarabine, we found no clear synergism for the platinum-based anti-neoplastic drug cisplatin with AZD1775 in DLBCL. Cisplatin treatment has many genotoxic effects, which include induction of monoadducts, inter- and intra-strand crosslinks, and/or DNA protein crosslinks [16]. Although cisplatin is currently used in many

cancer types, resistance to cisplatin monotherapy is a major issue [17]. Many studies have shown that WEE1 inhibition works synergistically with cisplatin, and is even able to overcome cisplatin resistance [11,12,18]. These observations were attributed to the abrogation of the cisplatin-induced G2/M arrest and abolishment of the cisplatin-induced CDC2 phosphorylation, through activation of the ATR-CHK1-WEE1 axis. Indeed, in SUDHL-10 pCDC2 protein levels appeared to steadily increase after cisplatin treatment, however these effects did not correlate with a significant additional cell death in combination treatment, and were not observed in SC-1. Moreover, we observed no G2/M arrest upon cisplatin treatment in DLBCL cell lines, whereas this has been described in other cancers and was suggested as part of the synergism mechanism for AZD1775 and cisplatin [12,18]. In addition, it must be noted that overall the DLBCL cell lines were much less sensitive to cisplatin treatment (average IC₅₀ 13 μ M) when compared to the reported (cisplatin-resistant) cell lines, and that much lower cisplatin treatment concentrations were used in the published combination experiments for head and neck cancer (1.5 μ M) [12], breast cancer (10 μ M) [11] and gastric cancer (2 μ M) [18]. This raises the question when cells should be labeled as “insensitive” or “resistant”, as this may differ greatly from cancer to cancer. In the current study, we observed that treatment of cisplatin-sensitive DLBCL cell lines (IC₅₀ $\leq$ 0.5 μ M) synergized with WEE1 inhibition, whereas treatment of cisplatin-insensitive cell lines (IC₅₀ $\geq$ 10 μ M cisplatin) did not, as a result of reduced WEE1 gene transcription. Based on the fact that DNA intercalation by cisplatin induces activation of the DNA damage response pathway, WEE1 would seem an obvious target to prevent DNA damage repair and induce catastrophic DNA damage. This is the case for cisplatin-sensitive cell lines, which indeed show an additive effect for cisplatin and AZD1775 combinational therapy. However, in cisplatin-insensitive cells we encounter a more sinister effect of cisplatin: a dramatic change in WEE1 protein and mRNA expression, most likely the result of this previously beneficial DNA intercalation preventing access of the transcriptional machinery. Several mechanisms have been described by which platinum-based compounds might inhibit gene transcription [19], of which stalling of RNA polymerase II [20] is often noted as a major contributor. Therefore, DNA intercalation by cisplatin treatment might be viewed as a double-edged sword: on the one hand it might lead to beneficial catastrophic DNA damage, whereas on the other hand it might dramatically alter expression of the protein targeted by the specific inhibitor.

Another often discussed factor in cancer therapy is the TP53 mutation status. Genotoxic agents such as cytarabine and cisplatin should work especially well in a TP53-mutated setting, as TP53-mutated cancers can no longer rely on the G1/S phase DNA damage repair checkpoint controlled by TP53, making them completely dependent on repair at the G2/M phase [21]. However, in our study cisplatin showed a lack of synergism in both TP53-mutated cell lines SUDHL-10 (c.994-1G>C) and U-2932 (c.527G>A), and in one of the two TP53 wild type cell lines, i.e. SC-1. Similarly, Osman et al. found no relation between TP53 status and synergism for AZD1775 and cisplatin [12]. Only for the TP53-mutated cell line SUDHL-2 (TP53: c.701A>C add protein change as well) synergism was observed, which was also the most cisplatin-sensitive cell line.

In conclusion, in this study we analyzed at the most commonly used second line chemotherapeutic and DNA damage-inducing agents cytarabine and cisplatin in DLBCL,

and found synergistic results for cytarabine combined with AZD1775, but dose-dependent antagonistic effects for cisplatin combined with AZD1775. These latter results seem in conflict with earlier publications, but we show a concentration dependent reduction of WEE1 protein activity and WEE1 gene transcription upon cisplatin treatment in cisplatin-insensitive cells. These results highlight the importance of thorough *in vitro* research for combination therapies, because seemingly rational treatment strategies might not be beneficial.

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Supporting information

Supplemental Table 1. DLCBL cell lines and their TP53 status and IC50 values for cytarabine, cisplatin and AZD1775. The sensitivity of eight DLBCL cell lines was determined by treatment of cells with cytarabine for 96 hours, cisplatin for 72 hours and AZD1775 for 72 hours. Cell viability was established using propidium iodide staining and flow cytometry analysis. Plotted is the average of n=3 experiments.

Cell line	Subtype	TP53 status	Cytarabine IC50 in μM	Cisplatin IC50 in μM	AZD1775 IC50 in nM
OCI-LY3	ABC	WT	0.6	1.5	357
U-2932	ABC	MUT	0.4	35	623
SUDHL-2	ABC	MUT	2	0.2	543
SUDHL-4	GCB	MUT	1	5	1835
SUDHL-5	GCB	WT	0.1	3	805
SUDHL-6	GCB	MUT	0.2	8	1503
SUDHL-10	GCB	MUT	0.2	5	608
SC-1	GCB	WT	0.5	50	412

Supplemental Table 2. Synergism calculation for cytarabine or cisplatin with AZD1775 in DLBCL cell lines. Shown are the expected and observed effect of the AZD1775 with cytarabine or cisplatin combination treatment with their p-values. Values are calculated from 3 individual experiments. Significant antagonistic combinations are depicted in dark red and significant synergistic combinations in dark green. Near significant or trend antagonistic combinations are depicted in light red and insignificant combinations are depicted in light green as they represent the additive combination treatments.

SUDHL-10					SC-1				U-2932				SUDHL-2			
Dose AZD1775	Dose Cytarabine	Expected	Observed	p-value	Dose Cytarabine	Expected	Observed	p-value	Dose Cytarabine	Expected	Observed	p-value	Dose Cytarabine	Expected	Observed	p-value
100 nM	100 nM	33	35	0,0159	100 nM	7	26	0,0019	100 nM	16	21	0,1020	100 nM	0	-1	0,8999
500 nM	100 nM	23	69	0,0007	100 nM	43	57	0,1008	100 nM	14	54	0,0897	100 nM	62	65	0,7365
100 nM	200 nM	37	40	0,2724	200 nM	19	37	0,0023	200 nM	24	34	0,0634	200 nM	-5	-2	0,5674
500 nM	200 nM	27	72	0,0015	200 nM	54	63	0,3278	200 nM	21	61	0,0117	200 nM	58	74	0,3207

SUDHL-10					SC-1				U-2932				SUDHL-2			
Dose AZD1775	Dose Cisplatin	Expected	Observed	p-value	Dose Cisplatin	Expected	Observed	p-value	Dose Cisplatin	Expected	Observed	p-value	Dose Cisplatin	Expected	Observed	p-value
100 nM	1 μM	25	16	0,0698	10 μM	6	9	0,4593	10 μM	18	25	0,1967	0,1 μM	18	11	0,3677
500 nM	1 μM	32	16	0,0517	10 μM	32	33	0,9231	10 μM	37	33	0,8044	0,1 μM	72	75	0,7874
100 nM	5 μM	47	46	0,9162	50 μM	51	51	0,7538	50 μM	76	79	0,3681	0,5 μM	66	61	0,3744
500 nM	5 μM	54	58	0,6697	50 μM	78	39	0,0038	50 μM	95	80	0,4238	0,5 μM	120	83	0,0700

Supplemental Figure 1. Combination therapy of cytarabine or cisplatin with AZD1775 in cell lines U-2932 and SUDHL-2. Efficiency of AZD1775 combined with (A) cytarabine for 96 hours and (B) cisplatin for 72 hours. Data was normalized to the control and plotted as the mean $\pm$ S.D. of $n=3$. Statistical analysis was performed with paired T-test. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$ and *** indicates $p \leq 0.001$.

Supplemental Figure 2. Cell cycle and γ H2AX analysis of cytarabine and AZD1775 combination therapy. (A) Representative cell cycle analysis of cell lines SUDHL-10 and SC-1 treated with cytarabine and AZD1775 for 24 hours. (B) Representative γ H2AX analysis of cell lines SUDHL-10 and SC-1 treated with cytarabine and AZD1775 for 24 hours.

Supplemental Figure 3. Cell cycle and γ H2AX analysis of cisplatin and AZD1775 combination therapy. (A) Representative cell cycle analysis of cell lines SUDHL-10 and SC-1 treated with cisplatin and AZD1775 for 24 hours. (B) Representative γ H2AX analysis of cell lines SUDHL-10 and SC-1 treated with cisplatin and AZD1775 for 24 hours.

Supplemental Figure 4. WEE1 protein and gene expression dosage effect of cytarabine treatment. (A) Representative western blot of WEE1, pCDC2 and γ H2AX protein levels in SUDHL-10 and SC-1 treated with increasing concentration of cytarabine for 24 hours. (B) mRNA expression of WEE1 in SUDHL-10 and SC-1 treated with increasing concentration cytarabine for 24 hours.

5

CHK1 isoforms determine response for CDK1 inhibitor and WEE1 inhibitor combination therapy in DLBCL

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Abstract

The current treatment of R-CHOP for diffuse large B-cell lymphoma (DLBCL) cures only 60% of patients, therefore novel therapeutic targets or treatment strategies are needed to improve therapy outcome. We recently showed that the WEE1 inhibitor AZD1775 resulted in DLBCL cell death as a result of increased DNA damage and premature mitotic entry. Here, we applied the popular paradigm of targeting multiple kinases in one pathway to improve the efficacy of AZD1775, by testing AZD1775 alongside CDK1 inhibitor RO3306.

We show that simultaneous treatment of cells with AZD1775 and RO3306 was synergistic in cell viability experiments in 50% of DLBCL cell lines, but induced strong antagonism in the other 50% of cases. In addition, combination of AZD1775 with CDK1/2 inhibitor roscovitine or CDK4/6 inhibitor palbociclib also resulted in strong antagonism in DLBCL cell lines. Analysis of DNA damage marker γ H2AX revealed antagonism was the results of reduced the levels of γ H2AX, thereby rescuing cells from AZD1775-induced DNA damage. Protein analysis of the DNA damage response pathway revealed treatment with RO3306 phosphorylated a long isoform of checkpoint kinase 1 (CHK1) at 56 kDa in antagonistic cells, whereas synergetic cells showed phosphorylation of a shorter CHK1 isoform at 45 kDa, indicating CHK1 determines therapy outcome.

Taken together, our results indicate that the success of the AZD1775 and CDK1 inhibitor combination treatment is determined by expression of the different phospho-CHK1 isoforms. This could have important implications for patient treatment, and therefore further research should be performed to investigate the effects and involvement of the CHK1 isoforms on therapy outcome.

Introduction

Genomic instability and activation of the DNA damage response are two hallmarks of diffuse large B-cell lymphoma (DLBCL) [1], the most common form of non-Hodgkin lymphoma. Despite increasing knowledge on the genetic profile of DLBCL, little advancement has been made in improving the current therapy regime, which consist of cyclophosphamide, vincristine, doxorubicin, prednisolone and the anti-CD20 antibody rituximab (R-CHOP). This current treatment cures roughly 60% of DLBCL patients, leaving many with relapse or refractory disease.

We recently discovered WEE1, a cell cycle regulator, as a novel target in DLBCL and demonstrated both single agent potential for WEE1 inhibitor AZD1775 [2] and synergism in combination with CHOP and radiation therapy (paper accepted for publication). To further enhance therapy response to AZD1775 and potentially reduce treatment dosage, we applied the paradigm of targeting multiple kinases in one pathway [3], by inhibiting both WEE1 and CDK1. During cell cycle progression, CDK1 forms a complex with cyclin A to stimulate S/G2 phase transition and forms a complex with cyclin B to allow cell cycle progression during G2/M phase, making it a key cell cycle regulator. Activity of CDK1 is controlled by the kinase WEE1, which inhibits CDK1 kinase activity through phosphorylation, and the CDC25 family of phosphatases, which remove WEE1's inhibiting phosphate group [4]. In addition to being involved in the same pathway, CDK1 was also independently one of our top 500 potential targets in DLBCL [2]. Pre-clinical research with CDK1 inhibitor RO3306 has shown to reduce proliferation and metastasis in neuroblastoma tumours [5] and enhance sensitivity to the multi-kinase inhibitor sorafenib in hepatocellular carcinoma [6]. Moreover, CDK1 inhibition has shown to be synthetic lethal with KRAS mutations in colorectal and pancreatic tumour cell models [7] and overcome apoptotic resistance in BRAF-mutant colorectal cancer when simultaneously treated with MEK/ERK inhibitors [8]. In addition, many CDK inhibitors such as seliciclib/roscovitine (CDK1/2 inhibitor) and palbociclib (CDK4/6 inhibitor) are currently being tested in clinical trials (NCT01333423; NCT02806648). Based on these data, CDK1 inhibition with RO3306 seems like a suitable treatment to test in combination with AZD1775, since both inhibitors target kinases which are involved in the same pathway, which fits in the current paradigm of cancer treatment [3].

Here, we showed that combination of AZD1775 and RO3306 induced either a synergistic or an antagonistic effect on cell viability, indicating DLBCL cells respond differently to treatment. Protein analysis of DNA damage response protein CHK1 revealed activation and phosphorylation at serine 345 of different isoforms. CHK1 was the determining factor for therapy outcome. Based on these data we propose further in-depth *in vivo* research for CDK1/2 inhibitors as therapy in combination with DNA damage inducing agents to determine the implications for clinical application.

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% fetal 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 checked on a regular basis. Cell of origin (COO), BCL-2 and MYC status was based on data from ATCC and DSMZ.

Compounds

The WEE1 inhibitor AZD1775 (#S1525, Houston, TX, USA), the CDK1 inhibitor RO3306 (#S7747), the CDK1/2 inhibitor roscovitine (#S1153) and the CDK4/6 inhibitor palbociclib (#S1579) were all acquired from Selleckchem. UV radiation was performed using UVP® CL-1000® Ultraviolet Crosslinkers.

Flow cytometry – Cell viability

For cell viability analysis, 0.2 x 10⁶ cells/mL were treated for the appropriate times, washed with 1% BSA/PBS and resuspended in propidium iodide solution (P4170, Sigma). Samples were processed on a BD FACSCalibur 2 and analysed with ModFit LT (Verity Software House). Experiments were performed three times.

Flow cytometry – cell cycle and γ H2AX

For cell cycle analysis, 0.2 x 10⁶ cells/ml were treated for the indicated time points, washed with 1% BSA/PBS and resuspended in solution containing 0.1% sodium citrate (A0158348, Merck, Kenilworth, New Jersey, United States), 0.01% propidium iodide (P4170, Sigma-Aldrich, St. Louis, Missouri, United States), 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 3 times.

For γ H2AX analysis, 0.2 x 10⁶ 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 analyzed using Kaluza 1.5 analysis software (Beckman). Experiments were performed 3 times.

Western blot

Western blot was performed as previously described (2). Staining with primary antibodies for anti-WEE1 (1:200, sc-5285 (B11), Santa Cruz Biotechnology, Dallas TX, USA), anti-phospho-CDC2 (Tyr15) (1:1000, #4539 (10A11), Cell Signaling Technology, Danvers, MA, USA), anti-phospho-Histone H2AX (Ser139) (1:1000, clone JBW301, Merck Millipore, Temecula, CA, USA) anti-phospho-CHK1 (Ser345) (1:1000, #2348 (133D3), Cell Signaling Technology), anti-phospho-RPA32 (Ser33) (1:1000, Bethyl, #A300-246A), anti-PAPR1 (1:1000, #9542, Cell Signaling Technology) and GAPDH (1:10,000; NovusBio) was done overnight at 4°C.

RT-qPCR

RNA was isolated from different DLBCL cell lines according to the protocol miRNeasy Mini Kit (Qiagen), followed by cDNA synthesis. 300 ng random primers hexamers and 10 mM dNTP-mix was added to 500 ng RNA. Samples were then incubated at 65°C for 5 min. After cooling on ice 5x first strand buffer, 10% v/v 0.1M dithiothreitol (DTT), 40 units/μl RNaseOUT and 200 units Superscript II was added to the samples. cDNA was synthesized using the following programme; 10 min at 25°C, 50 min at 42°C and 15 min at 70°C.

Table 1. Primers and probes for CHK1 RT-qPCR.

Gene	Forward primer	Reversed primer	Probe
CHK1	5' - GGTGCCTATG GAGAAGTTCAA - 3'	5' - TCTACGGCAC GCTTCATATC - 3'	5' - CAATCTTCAC TGCGACTGCTTCTCAG -3'
CHK1-S	5' - GTGCAAACCC TGGGAGAA - 3'	5' - CTCTGAGCAT CTGGTTCAGG - 3'	5' - TGCCTATGTC TGGCTTCTCCATAGGC -3'
HPRT	5' - GGCAGTATAA TCCAAAGATGGTCAA - 3'	5' - GTCTGGCTTA TATCCAACACTTCG - 3'	5' - CAAGCTTGCT GGTGAAAAGGACCCC -3'

Statistical analysis

Data were analyzed using Graphpad PRISM (version 5.0) software and tested for significant differences with a paired T-test. Correlation was analyzed using Spearman's correlation. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$ and *** indicates $p \leq 0.001$.

Results

DLBCL cell lines show either a synergistic or an antagonistic response to CDK1i and WEE1i combination therapy

Both WEE1 inhibition and CDK1 inhibition are currently being tested as (mono)therapy in several types of cancer. To evaluate the efficiency of a combination therapy, we treated eight DLBCL cell lines with WEE1 inhibitor AZD1775 and CDK1 inhibitor RO3306 for 72 hours (Figure 1A). In half of our DLBCL cell line panel (SUDHL-10, SUDHL-5, SUDHL-6 and SUDHL-2), the combination of AZD1775 and RO3306 resulted in a synergistic reduction in cell viability (Supplemental table 1), where addition of AZD1775 enhanced cell death with on average 40%. However, in other cell lines (U-2932, SC-1, OCI-LY3 and SUDHL-4) the same combination of AZD1775 and RO3306 had no (additional) effect on cell viability or rescued the AZD1775-induced cell death, making the combination antagonistic (Supplemental table 1). These outcomes were not related to a specific cell or origin (COO) or TP53 mutations status (Supplemental table 2).

Figure 1. Combination of RO3306 (CDK1i) with AZD1775 (WEE1i) in DLBCL cell lines. (A) Cell viability curves of eight DLBCL cell lines treated with CDK1 inhibitor RO3306 and WEE1 inhibitor AZD1775 for 72 hours. Data was normalized to the control and plotted as the mean $\pm$ S.D. of $n=3$ experiments. (B) Analysis of DNA damage marker γ H2AX after treatment with RO3306 and AZD1775 for 24 hours ($n=3$).

Analysis of DNA damage marker γ H2AX confirmed that in cell lines where the combination of AZD1775 and RO3306 showed synergism, the combination significantly

enhanced DNA damage levels with on average 20% (Figure 1B). In contrast, cell lines where the combination of AZD1775 and RO3306 showed antagonism, the same combination resulted in a strong rescue of DNA damage levels. To determine the factor(s) that could regulate either the synergistic or antagonistic outcome, we analyzed the cell viability data for RO3306 and AZD1775 and found that synergistic cell lines were twice as sensitive to RO3306 (mean IC₅₀ 6 μ M; $p=0.0366$) compared to antagonistic cell lines (mean IC₅₀ 11 μ M) (Supplemental figure 1A), while no differences were observed for AZD1775 (Supplemental figure 1B). In addition, cell cycle analysis showed treatment after CDK1 blocking with RO3306 increased the percentage of G2/M phase cells as a result of cell cycle arrest induced by CDK1 inhibition, however no significant differences were observed between synergistic or antagonistic cell lines (Supplemental figure 2). Taken together, these results show that AZD1775 combined with RO3306 can induce either a synergistic or antagonistic effect on cell viability of DLBCL cell lines, which is likely caused by enhanced or reduced DNA damage levels, respectively.

Figure 2. Combination of roscovitine (CDK1/2i) or palbociclib (CDK4/6i) with AZD1775 (WEE1i) in DLBCL. (A) Cell viability analysis of DLBCL cells treated with roscovitine combined with AZD1775 in cell lines SUDHL-10, SUDHL-6, SC-1 and U-2932 for 72 hours. Data was normalized to the control and plotted as the mean $\pm$ S.D. of $n=3$ experiments. (B) Analysis of DNA damage marker γ H2AX after treatment with roscovitine and AZD1775 for 24 hours ($n=3$). (C) Cell viability analysis of cells treated with palbociclib combined with AZD1775 for 72 hours. (D) Analysis of DNA damage marker γ H2AX after treatment with palbociclib combined with AZD1775 for 24 hours ($n=3$).

To evaluate whether other CDK inhibitors could have a similar dual effect like observed for RO3306, we looked at cell viability and DNA damage levels in cells treated with AZD1775 combined with CDK1/2 inhibitor roscovitine or CDK4/6 inhibitor palbociclib. Since CDK1/2 are involved in the regulation of the S/G2/M phase and CDK4/6 in regulation of the G1 phase, these data will help elucidate if the stage of cell cycle arrest plays a role in determining

therapy outcome when combined with AZD1775. Cell viability results in two representative “synergistic (S)” cell lines SUDHL-6 and SUHDL-10 showed only minor levels of enhanced cell death when roscovitine was combined with AZD1775, whereas significant antagonism with respect to cell viability was observed in the two representative “antagonistic” cell lines SC-1 and U-2932 (Figure 2A). In addition, DNA damage analysis revealed mild enhanced cell death in SUHDL-10 (S) and SUDHL-6 (S) at lower concentration of 0.5 μ M AZD1775, whereas the combination of roscovitine with 1 μ M AZD1775 resulted in reduced γ H2AX levels, indicating antagonism (Figure 2B). In line with previous cell viability data for SC-1 and U-2932 (Figure 2A), combination of roscovitine with AZD1775 resulted in strong reduction of γ H2AX levels (Figure 2B). These results demonstrate that CDK1/2 inhibitor roscovitine has a very different effect when combined with AZD1775 compared to CDK1 inhibitor RO3306, which potentially induces strong synergism in 50% of the cells. In contrast, combination of AZD1775 with palbociclib showed antagonism in both cell viability (Figure 2C) and DNA damage analysis (Figure 2D) in all four cell lines. Together, these data show that CDK inhibitors are likely not suitable to combine with AZD1775, as they result in strong antagonism in DLBCL cell lines in most cases.

DLBCL cell lines express different phospho-CHK1 isoforms upon CDK1 inhibition

Based on previous data, we wanted to further investigate how the combination of AZD1775 and RO3306 can results in both synergistic and antagonistic on cell viability, and how RO3306 differs from the other CDK1 inhibitors. To this end we first performed protein analysis of the DNA damage response pathway in “synergistic” cell line SUDHL-5 treated with AZD1775, RO3306, roscovitine, palbociclib and as positive control UV radiation (Figure 3).

Figure 3. Protein analysis of DNA damage response proteins. Analysis of DNA damage response proteins pCHK1, pCHK2, WEE1, pCDC2, γ H2AX, pRPA, (cleaved) PARP and H3.

Treatment of cells with WEE1 inhibitor AZD1775 induced reduction of WEE1 and its downstream target phospho-CDC2 (Tyr15). In addition, treatment induced activation of DNA damage-related proteins γ H2AX, phospho-RPA and marker of apoptosis cleaved PARP. For CDK1 inhibitor RO3306, no changes were observed in the phosphorylation levels of CDC2, however treatment resulted in strong activation of γ H2AX (double-stranded DNA breaks) and activation of cleaved PARP. In addition, treatment with RO3306 resulted in activation of phospho-CHK1, however at a much lower molecular weight of 45 kDa, instead of the conical 56 kDa, as observed after UV radiation. For CDK1/2 inhibitor roscovitine, treatment activated both γ H2AX and cleaved PARP, but not phospho-CHK1. Similarly, CDK4/6 inhibitor palbociclib induced both phospho-RPA and cleaved PARP in the absence of CHK1 activation, unlike RO3306. Moreover, treatment with palbociclib significantly reduced protein levels of total CHK1, indicating less potential for CHK1 activation. These data demonstrate the different effects induced by different CDK1 inhibitors on the DNA damage response pathway, and how they potentially might alter the outcome of combination therapies with DDR inhibitors.

Figure 4. Western blot analysis of pCHK1 protein levels after treatment with CDK1i and WEE1i. (A) Protein analysis of phospho-CHK1 (Ser345), WEE1, phospho-CDC2 (Tyr15) and γ H2AX after treatment with CDK1 inhibitor RO3306 and/or WEE1 inhibitor AZD1775 for 24 hours in synergistic cell lines SUDHL-10 and SUDHL-5. (B) Protein analysis of phospho-CHK1, WEE1, phospho-CDC2 and γ H2AX after treatment with RO3306 and/or AZD1775 for 24 hours in antagonistic cell lines U-2932 and SC-1.

Based on previous data, we further tested the effect of AZD1775 combined with RO3306 on the phosphorylation of CHK1 in two representative synergistic (SUDHL-10 and SUDHL-5) and two antagonistic (U-2932 and SC-1) DLBCL cell lines (Figure 4). Protein analysis showed activation of phospho-CHK1 in synergistic cell lines SUDHL-5 and SUDHL-10 was observed at a protein size of 45 kDa upon treatment with RO3306, whereas treatment with AZD1775 induced no phospho-CHK1 activation in cell line SUDHL-5 and a weak band of phospho-CHK1 at a protein size of 56 kDa in cell line SUDHL-10 (Figure 4A). In contrast, activation

of phospho-CHK1 upon treatment with RO3306 was not observed in antagonistic cell lines SC-1 and U-2932, whereas treatment with AZD1775 induced strong activation of phospho-CHK1 at a protein size of 56 kDa (Figure 4B). Protein levels of γ H2AX showed a similar activation pattern as the phospho-CHK1 protein expression and previous flow cytometry data in both synergistic and antagonistic cells lines (Figure 1B), indicating phospho-CHK1 was activated in response to DNA damage induction.

Taken together, these data indicate that cells that show synergism upon the AZD1775 and RO3306 combination treatment activate a shorter, and perhaps less functional isoform of phospho-CHK1, resulting in inefficient DNA damage repair and enhanced cell death. At the same time, cells that show an antagonistic response to AZD1775 and RO3306 combination treatment activate a long isoform of phospho-CHK1 that likely is able to efficiently activate pathways for repair, thereby reducing DNA damage in the combination treatment.

Gene expression of CHK1 short is very low compared to CHK1 long in DLBCL cell lines

Figure 5. Gene expression of CHK1 and CHK1-S in DLBCL cell lines. (A) Gene expression of the CHK1 long and short isoform in DLBCL cell lines that showed synergism (left) or antagonism (right) for the combination of AZD1775 with RO3306. (B) Ratio of gene expression levels of CHK1 long to CHK1 short in DLBCL cell lines. (C) Gene expression of CHK1 long and short isoforms in cell lines SUDHL-10, SUDHL-5, SC-1 and U-2932 treated with AZD1775, RO3306 or combined for 24 hours.

Based on our protein analysis, we established that the two CHK1 isoforms are actively phosphorylated on serine 345 in response to different treatments. However, analysis of total CHK1 protein showed little expression of the short CHK1 isoform. Therefore, we checked if the CHK1 gene expression levels were also altered by CDK1 inhibitor treatment.

Gene expression of the long CHK1 (CHK1) and short CHK1 (CHK1-S) isoforms in the DLBCL cell line panel showed expression of CHK1-S was significantly (~10-fold) lower compared to CHK1 (Figure 5A), which would explain the undetectable expression levels of the short CHK1 isoform in the western blot. Comparison of the different cell lines showed there were no differences between synergistic and antagonistic cell lines in the level of CHK1-S expression levels or the CHK1/CHK1-S ratio (Figure 5A-B). Treatment of cells with RO3306 alone or in combination with AZD1775 induced an increase in gene expression levels of both CHK1 and CHK1-S, and was observed in both synergistic and antagonistic cells lines (Figure 5C and Supplemental figure 3). Based on these data, it seems phosphorylation of the different CHK1 isoforms, and not expression of the CHK1 isoforms, is the critical difference between synergistic or antagonistic outcome.

CDK1i treatment, but not WEE1i, HU, cytarabine or UV activate the phospho-CHK1 short isoform

Next, we investigated whether other forms of DNA damage inducing compounds could also activate the short isoform of phospho-CHK1 in the representative cells lines SUDHL-10 (synergism) and SC-1 (antagonism). Cells were treated for 6 or 24 hours with hydroxy urea (HU), a known inducers of replication stress [9], and the anti-metabolic agent cytarabine, known for its interference with DNA synthesis [10], alongside RO3306 and AZD1775 as positive and negative controls. Protein analysis revealed activation of the short phospho-CHK1 isoform was only present in SUDHL-10 cells treated with RO3306 after 24 hours (Figure 6A). Activation of the long CHK1 isoform was observed after 6 hours of treatment with AZD1775, but was absent at 24 hours, despite the presence of γ H2AX. Treatment with hydroxy urea and cytarabine activated the long phospho-CHK1 isoform but not the short isoform, and showed induction of γ H2AX. Analysis of total CHK1 protein expression showed strong presence of the long CHK1 isoform, but expression of the CHK1 short isoform was only observed in cells treated with RO3306 for 24 hours. In cell line SC-1, treatment with RO3306 activated neither the short nor the long isoform of phospho-CHK1, whereas AZD1775, hydroxy urea and cytarabine all activated the long phospho-CHK1 isoform and induced expression γ H2AX (Figure 6B). In all treatments, activation of (long) phospho-CHK1 in SC-1 was more pronounced after 6 hours treatment compared to 24 hours treatment. Analysis of total CHK1 protein showed only expression of the long CHK1 isoform, which is in concordance with activation of only the long phospho-CHK1 isoform. Similar results were obtained when cell lines SUDHL-10 and SC-1 were treated with UV radiation, which also activated the long, but not the short phospho-CHK1 isoform (Supplemental figure 4). In addition, combination of UV with RO3306 showed a similar pattern as AZD1775 combined with RO3306, in which cell lines SUDHL-10 and SUDHL-5 showed synergism and cell lines

SC-1 and U-2932 showed antagonism for the combination (Supplemental figure 5). These results demonstrate a unique activation pattern of isoforms for phospho-CBK1, which is both treatment specific but also timing dependent.

Figure 6. Western blot analysis of pCHK1 protein levels after treatment with chemotherapeutic compounds. (A) Protein analysis of phospho-CBK1 (Ser345), WEE1, phospho-CDC2 (Tyr15) and yH2AX after treatment with 10 μM CDK1 inhibitor RO3306, 1 μM WEE1 inhibitor AZD1775, 3 mM hydroxy urea (HU) or 1 μM cytarabine in cell line SUDHL-10. Samples were harvested after 6 and 24 hours. (B) Protein analysis of phospho-CBK1, WEE1, phospho-CDC2 and yH2AX after treatment with 10 μM CDK1 inhibitor RO3306, 1 μM WEE1 inhibitor AZD1775, 3 mM hydroxy urea (HU) or 1 μM cytarabine in cell line SC-1. Samples were harvested after 6 and 24 hours.

Discussion

Many CDK inhibitors are currently being tested as possible anti-cancer therapeutics, including seliciclib/roscovitine (CDK1/2 inhibitor; NCT01333423), palbociclib (CDK4/6 inhibitor; NCT02806648), voruciclib/P1446A-05 (CDK4i; NCT01841463) and AT7519M (CDK1/2/4/5/9i; NCT01652144). However, these therapies will most likely be used in combination with other (existing) therapies, warranting the need for proper combination studies. Here, we show the potential danger of combining CDK1 inhibitor RO3306 with DNA damage-inducing treatments, which can induce both synergistic and antagonistic effects (Figure 1). Our data showed that one of the determining factors of therapy outcome could be the phosphorylation of either a short or long isoform of the checkpoint kinase CHK1 (Figure 4). CHK1 is activated by the DNA damage response kinase ATR, which mostly respond to single stranded DNA damage or replication stress [11]. Activation of CHK1 results in downstream inhibition of cell cycle progression, through inhibition of CDC25A and CDC25C, and subsequent activation of WEE1 [12]. At the same time, CHK1 is also able to prevent apoptosis, although the exact mechanism behind this phenomenon is still unknown [13]. Research has shown the existence of two different isoforms of CHK1, a (normal) long CHK1 isoform of 56 kDa and a short n-terminally truncated CHK1 splice variant with a size of ~43 kDa, which is missing part of its kinase domain [14-16]. This short CHK1 isoform was highly

expressed in cancer tissue compared to control healthy tissue, and the presence of the short CHK1 isoform was strongly associated with disease progression in patient samples of ovarian cancer and prostate cancer [16]. Moreover, it has been suggested that through direct interaction of the short and long isoform, activity of the long isoform was diminished [16,17]. However, these experiments focused mainly on the presence or absence of the short and long CHK1 proteins itself, and did not further study the CHK1 phosphorylation sites. Here, we show for the first time that phosphorylation at site serine 345 of these isoforms is differently activated in response to therapeutic treatment, and that they may determine therapy outcome in DLBCL cell lines treated with CDK1 inhibitor RO3306 combined with DNA damage inducing drugs. This would pose a serious problem for patient treatment, and therefore further studies with *in vivo* or PDX models should be performed to further validate these outcomes before we can properly determine the clinical implications. In addition, the question remains 1) why the alternative isoform phosphorylation is active in specific cells but not in others, and 2) how specific treatment is able to activate this alternative isoform phosphorylation, since the short CHK1 isoform is likely less functional and therefore less beneficial for cellular survival.

In addition, we showed combination of AZD1775 with CDK4/6 inhibitor palbociclib always induced strong antagonistic effects in DLBCL (Figure 2). These data are in line with research from Roberts et al., who showed co-administration of palbociclib with carboplatin, doxorubicin, etoposide, camptothecin or paclitaxel all resulted in reduced levels of γ H2AX foci, indicating palbociclib reduced levels of DNA damage induced by these chemotherapeutic drugs [18]. Similar to CDK1/2 inhibitors, it appears the CDK4/6 inhibitor palbociclib can block effectivity of DNA damage-inducing agents, resulting in strong antagonism. However, where the effects of CDK1/2 inhibition are possibly dose-dependent, CDK4/6 inhibition seem to always induce strong antagonism. This indicates an important difference in perhaps the underlying mechanism behind this phenomenon or the importance of the different cell cycle stage at which these events are taking place. Further research would be needed to elucidate the differences and/or similarities between the different CDK proteins and how they are affected by inhibitor treatment.

In conclusion, based on the current evidence we propose a model for the possible outcomes when CDK1 inhibitor RO3306 is combined with DNA damage-inducing compounds (Figure 7A) and a model when two (or more) DNA damage-inducing compounds are combined (Figure 7B). In the first situation (Figure 7A), DNA damage induction combined with RO3306 allows cells time for repair or survival by arresting cells at the G2/M phase. However, DNA damage repair and avoidance of apoptosis is only achieved if cells are able to activate the long isoform of CHK1, which contains the full-length kinase domain for activation of downstream targets. If through alternative activation the short CHK1 isoform is phosphorylated, cells are unable to induce repair and avoid apoptosis, resulting in (enhanced) cell death. If cells are treated with a combination of two (or more) DNA damage-inducing compounds (Figure 7B), cells experience a double hit of DNA damage, making it harder to cope with cellular stress and detain apoptosis. At the same time, cells are not automatically arrested to allow repair. Therefore, even though a full length and functional CHK1 protein is activated, cells are unable to cope with the extreme levels of damage and stress, resulting in cell death.

Figure 7. Schematic overview of the CHK1 isoforms and their involvement in DNA damage repair and apoptosis. (A) In the situation when DNA damage inducing treatments are combined with CDK1 inhibitor RO3306 (left panel), the cell cycle arrest induced by Ro3306 has the potential to allow DNA damage repair, thereby avoiding apoptosis. However, proper repair can only be performed when cells activate the long phospho-CHK1 isoform, whereas activation of the short phospho-CHK1 isoform results in apoptosis. (B) In the situation when two (or more) DNA damage inducing treatments are combined (right panel), the extent of DNA damage is too severe to repair and therefore cells will undergo apoptosis, despite activation of the long CHK1 isoform.

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Supporting information

Supplemental Table 1. Combination index (CI) for RO3306 combined with AZD1775. The combination index (CI) was based on cell viability data for RO3306 combined with AZD1775 after 72 hours of incubation. CI was calculated using the Chou-Talalay method. Numbers in red are classified as antagonistic ($CI > 1$), in white as additive ($CI = 1$) or in green as synergistic ($CI < 1$).

SUDHL-10		
RO3306/AZD1775	100 nM	500 nM
2 µM	2,0	0,5
4 µM	3,0	0,6
6 µM	0,7	0,8
8 µM	0,8	0,9
10 µM	1,0	1,1

SUDHL-5		
RO3306/AZD1775	100 nM	500 nM
2 µM	39	1,2
4 µM	1,2	0,8
6 µM	0,8	0,9
8 µM	1,0	1,1
10 µM	1,1	1,3

SUDHL-6		
RO3306/AZD1775	100 nM	500 nM
2 µM	1,2	1,3
4 µM	1,0	1,1
6 µM	0,7	1,1
8 µM	0,8	1,2
10 µM	0,9	1,3

SUDHL-2		
RO3306/AZD1775	100 nM	500 nM
2 µM	1,3	0,9
4 µM	0,7	0,7
6 µM	0,7	0,8
8 µM	0,9	0,9
10 µM	0,9	0,9

SC-1		
RO3306/AZD1775	100 nM	500 nM
2 µM	2,7	1,1
4 µM	0,8	1,2
6 µM	0,9	1,5
8 µM	1,2	1,7
10 µM	1,4	2,0

U2932		
RO3306/AZD1775	100 nM	500 nM
2 µM	1,3	0,8
4 µM	0,6	1,1
6 µM	1,0	1,7
8 µM	1,3	1,9
10 µM	1,5	2,1

SUDHL-4		
RO3306/AZD1775	100 nM	500 nM
2 µM	94	101
4 µM	1,6	1,9
6 µM	1,1	1,4
8 µM	1,1	1,4
10 µM	1,2	1,5

OCI-LY3		
RO3306/AZD1775	100 nM	500 nM
2 µM	1,1	1,2
4 µM	1,0	1,5
6 µM	1,1	1,9
8 µM	1,4	2,1
10 µM	1,7	3,7

Supplemental Table 2. Overview of DLBCL cell lines characteristics. Overview of the eight DLBCL cell lines with their corresponding cell of origin (COO) classification, TP53 mutation status, IC50 value for RO3306 and AZD1775 and response to the AZD1775 and RO3306 combination treatment.

Cell line	Subtype	TP53 status	RO3306 (μ M)	AZD1775 (nM)	Combination response
SUDHL-10	GCB	MUT	9,0	930	Synergism
SUHDL-5	GCB	WT	3,8	558	Synergism
SUDHL-6	GCB	MUT	6,0	462	Synergism
SUDHL-2	ABC	MUT	4,7	520	Synergism
SC-1	GCB	WT	14,4	778	Antagonism
U-2932	ABC	MUT	9,1	710	Antagonism
OCI-LY3	ABC	WT	9,2	225	Antagonism
SUDHL-4	GCB	MUT	9,3	1140	Antagonism

Supplemental Figure 1. Comparison of IC50 values between synergistic and antagonistic DLBCL cell lines. (A) Comparison between IC50 values for RO3306 between cell lines which showed synergism for the RO3306 and AZD1775 combination (SUDHL-2, SUDHL-5, SUDHL-6 and SUDHL-10) and cell lines which showed antagonism for the RO3306 and AZD1775 combination (SC-1, U-2932, OCI-LY3 and SUDHL-4). (B) Comparison between IC50 values for AZD1775 between cell lines which showed synergism for the RO3306 and AZD1775 combination (SUDHL-2, SUDHL-5, SUDHL-6 and SUDHL-10) and cell lines which showed antagonism for the RO3306 and AZD1775 combination (SC-1, U-2932, OCI-LY3 and SUDHL-4).

Supplemental Figure 2. Cell cycle analysis of DLBCL cell lines treated with CDK1i and WEE1i. Flow cytometry cell cycle analysis data of eight DLBCL cell lines treated for 24 hours with CDK1 inhibitor RO3306 and WEE1 inhibitor AZD1775. Plotted are the mean of n=3 experiments.

Supplemental Figure 3. Gene expression ratio of CHK1-S and CHK1 in DLBCL cell lines. Gene expression ratio was calculated by dividing the short CHK1 isoform (CHK1-S) by the normal CHK1 isoform (CHK1). Plotted are the results for two synergistic cell lines (SUDHL-10 and SUHDL-5) and two antagonistic cell lines (SC-1 and U-2932).

Supplemental Figure 4. Western blot analysis of pCHK1 protein levels after treatment with UV radiation. Protein analysis of phospho-CHK1 (Ser345), CHK1, WEE1, phospho-CDC2 (Tyr15), γH2AX, phospho-RPA (Ser33) and PARP after treatment with 20 J/m² UV radiation in cell line SUDHL-10 and SC-1. Samples were harvested after 6 and 24 hours.

Supplemental Figure 5. Combination of CDK1i with UV radiation in DLBCL cell lines. Cell viability curves of four DLBCL cell lines treated with CDK1 inhibitor RO3306 and UV radiation for 72 hours. Data was normalized to the control and plotted as the mean.

WEE1 inhibition enhances anti-apoptotic dependency as a result of premature mitotic entry and DNA damage

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Abstract

Genomically unstable cancers are dependent on specific cell cycle checkpoints to maintain viability and prevent apoptosis. The cell cycle checkpoint protein WEE1 is highly expressed in genomically unstable cancers, including diffuse large B-cell lymphoma (DLBCL). Although WEE1 inhibition effectively induces apoptosis in cancer cells, the effect of WEE1 inhibition on anti-apoptotic dependency is not well understood. We show that inhibition of WEE1 by AZD1775 induces DNA damage and pre-mitotic entry in DLBCL, thereby enhancing dependency on BCL-2 and/or MCL-1. Combining AZD1775 with anti-apoptotic inhibitors such as venetoclax (BCL-2i) or S63845 (MCL-1i) enhanced sensitivity in a cell-specific manner. In addition, we demonstrate that both G2/M cell cycle arrest and DNA damage induction put a similar stress on DLBCL cells, thereby enhancing anti-apoptotic dependency. Therefore, genotoxic or cell cycle disrupting agents combined with specific anti-apoptotic inhibitors may be very effective in genomic unstable cancers such as DLBCL and therefore warrants further clinical evaluation.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is a genomically unstable cancer with multiple low-frequency mutations, somatic copy number alterations, and chromosomal translocations [1]. To survive in such a genetically hazardous setting, DLBCL cells rely on cell cycle checkpoints, DNA repair, and anti-apoptotic proteins [2,3]. We have recently demonstrated that the cell cycle regulator WEE1 is highly expressed in DLBCL and is a relevant target for therapy [4]. WEE1 acts through phosphorylation of cyclin-dependent kinase 1 (CDK1/CDC2), which blocks the cell cycle at G2/M to allow time for DNA damage surveillance [5]. Under normal circumstances, factors such as cell cycle disruption and DNA damage result in induction of (intrinsic) apoptosis [6–10]. Indeed, blocking of WEE1 activity with AZD1775 results in apoptosis in solid cancers [11], lymphoma [4], and leukaemia [12,13]. In order to survive intrinsic apoptosis, DLBCL and many other cancers induce upregulation of various anti-apoptotic proteins, leading to apoptosis resistance, resulting in a cellular reliance on anti-apoptotic proteins commonly termed 'anti-apoptotic dependency' [14,15]. Since AZD1775 is very effectively able to induce intrinsic apoptosis, we aimed to investigate whether AZD1775 could alter the anti-apoptotic dependency and enhance the sensitivity of DLBCL cells to anti-apoptotic inhibitors (a.k.a., BH3 mimetic drugs).

The intrinsic apoptotic pathway is regulated by a complex interaction of pro- and anti-apoptotic proteins at the surface of mitochondria. Cells undergo apoptosis when this protein complex is balanced towards pro-apoptotic proteins (e.g., BIM (pro-apoptotic effector), BAD, NOXA, PUMA, BMF, HRK (pro-apoptotic sensitizers)). On the other hand, when apoptotic dependency shifts towards anti-apoptotic proteins (e.g., BCL-2, BCL-XL, BCL-W, and MCL-1) cells can avoid apoptosis [16]. Many studies in both cell lines and primary patients' samples have demonstrated the (clinical) potential of BH3 profiling, a technique that can identify a selective dependency on individual pro- or anti-apoptotic proteins in this complex network [17]. With this method, cells are exposed to BH3 peptides which enter the mitochondria and interact with anti-apoptotic proteins, resulting in a mitochondrial outer membrane permeabilization (MOMP) which can be measured using the JC-1 dye or as cytochrome-c release. Dynamic BH3 profiling (dBH3) is an *ex vivo*, clinically applicable variant of BH3 profiling which measures early changes in the apoptotic signalling cascade at the mitochondrial level, whether induced in cancer cells by treatment with classical chemotherapy or by specific anti-cancer agents [18–20].

In the present study we showed that WEE1 inhibitor AZD1775 induces cell death through apoptosis. In addition, we showed that AZD1775 significantly enhanced the dependency on anti-apoptotic proteins and enhanced sensitivity to BH3 mimetic drugs in DLBCL cells in a cell-specific manner. Similar changes in the anti-apoptotic dependency were induced by DNA damage and cell cycle arrest independently. Based on these results, we predict that AZD1775 and other similar drugs are very suitable to combine with BH3 mimetic drugs for the treatment of cancer.

Materials and Methods

Cell Lines and Culture Conditions

The DLBCL cell lines U-2932 (ABC), SUDHL-2 (ABC), SUDHL-4 (GCB), and SC-1 (GCB) were cultured in suspension in Roswell Park Memorial Institute medium 1640 (RPMI 1640; Lonza BioWhittaker, Walkersville, MD, USA) with 10% fetal 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 (ABC), SUDHL-5 (GCB), SUDHL-6 (GCB), and SUDHL-10 (GCB) 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 checked on a regular basis. Cell line characteristics are given in Table S1.

Patient Material and METC Statement

Patient material was acquired in accordance with international regulations and professional guidelines (the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice). Material used in this project was obtained from anonymous rest material. The medical ethics review board waives the need for approval if rest material is used, under the law in the Netherlands and waives the need for informed consent when patient anonymity is assured.

BH3 Profiling—Plate-Based Assay

Cells were incubated at 0.5×10^6 cells/mL for 18 h with AZD1775 (WEE1 inhibitor, Selleckchem No.S1525, Houston, TX, USA) (Figure S8), palbociclib (CDK4/6 inhibitor, Selleckchem No.S1116), RO3306 (CDK1 inhibitor, Selleckchem No.S7747), roscovitine (CDK2 inhibitor, Selleckchem No. S1153), nocodazole (M1404, Sigma Aldrich, St. Louis, MO, USA), or ultraviolet (UV) radiation (IBL 637, CisBioInternational, Gif-sur-Yvette, France). After incubation, cells were washed with mannitol experimental buffer (MEB) (150 mM D-mannitol (M9647, Sigma Aldrich), 10 mM HEPES (H3375, Sigma Aldrich), 50 mM KCl (1.04936, Merck, Darmstadt, Germany), 20 nM EGTA (E4378, Sigma Aldrich), 20 nM EDTA (11280, Serva Electrophoresis, Heidelberg, Germany), 0.1% BSA (11930, Serva Electrophoresis), 5 mM succinate (1.00682, Merck) in dH₂O, pH 7.5) and resuspended at 3.2×10^6 cells/mL in MEB. A cell suspension was mixed 1:1 with 4 μ M JC-1 permeabilization/staining solution ((ENZ-52304, Enzo Life Sciences, Farmingdale, NY, USA), 0.004% digitonin (1500 643, Boehringer Mannheim, Mannheim, Germany), 20 mM β -mercaptoethanol (8.05740, Merck) and 40 μ g/mL oligomycin (O4876, Sigma Aldrich) prepared in MEB) and incubated at room

temperature in the dark for 10 min. BIM, PUMA, BAD, NOXA, MS1, HRK, BMF, and PUMA2A (JPT Peptide Technologies, Berlin, Germany) were prepared in MEB in a black flat-bottom non-treated polystyrene 96-well plate (3915 Costar, Corning Incorporated, Kennebunk, ME, USA). Peptide sequences used for the assay were identical to those described in Ryan and Letai [18]. Plates were either used directly or sealed (Silverseal sealer ref 676090, Greiner-Bio-One, Frickenhausen, Germany), frozen at -80°C and thawed for 1 h at room temperature before use. Cells in permeabilization/staining solution were added to the plate 1:1 at a final volume of 100 μL and shaken for 15 s, followed by measurement of fluorescence (excitation 545 nm, emission 590 nm) every 5 min for 2 h at 30°C (Varioskan). All experiments were performed in triplicates or quadruplicate. The area under the curve (AUC) was calculated as a percentage of mitochondrial outer membrane permeabilization (MOMP) and normalized to PUMA2A (negative control) and FCCP (positive control) with the formula: $1 - ((\text{AUC sample} - \text{AUC FCCP}) \div (\text{AUC PUMA2A} - \text{AUC FCCP})) \times 100\%$. The dynamic BH3 profile (ΔMOMP) was calculated by subtracting the percentage treated MOMP from percentage untreated MOMP. Based on the specific interacting partners of the BH3 peptides [18], the cell specific anti-apoptotic dependency of cells can be established. BH3 profiling with a mean $\Delta\text{MOMP} \geq 20\%$ were classified as biologically relevant, even if they were not always statically significant. ΔMOMP changes for the BIM peptide indicates cells have become more primed for apoptosis or closer to their apoptotic threshold. Similarly, ΔMOMP changes for NOXA, HRK, or BAD indicate cells have become more dependent on MCL-1, BCL-XL, or BCL-2/XL/W, respectively. Together, these data can predict on which anti-apoptotic protein cells are dependent, and how this changes upon inhibitor treatment.

BH3 Profiling—Flow Cytometry-Based Assay

Peripheral blood mononuclear cells were isolated from a DLBCL patient, washed with 10% FBS RPMI, incubated at 1.0×10^6 cells/mL for 18 h with 1 μM AZD1775 (WEE1 inhibitor, Selleckchem) in RPMI medium supplemented with 10% fetal bovine serum, 1% Penicillin-Streptomycin, and 1% glutamine. After incubation, cells were washed with 1% BSA (11930, Serva) in PBS and incubated with CD19-APC antibody (IQP-106A, IQ Products, Groningen, The Netherlands) for 30 min at 4°C . After staining, cells were washed with and suspended in MEB at $3\text{--}5 \times 10^6$ cells/mL. Cells were incubated with peptides in 0.001% digitonin at 25°C for 60 min. The reaction was stopped by addition of 2% formaldehyde (104005, Merck) in PBS and cells were fixed in 2% formaldehyde for 20 min at room temperature. Cells were washed in 1% BSA in PBS and stained overnight for intracellular cytochrome-c in PBS containing 1% BSA in PBS, 0.2% Tween 20 (P7949 Sigma Aldrich) and 1:400 Alexa Fluor 488 anti-cytochrome-c antibody (612308, BioLegend 9727 Pacific Heights Blvd. San Diego, CA 92121, USA) at 4°C . Before analysis, cells were washed with 1% BSA in PBS and flow cytometry analysis was performed on a FACSCalibur (BD Biosciences, 1 Becton Drive, Franklin Lakes, NJ 07417-1880, USA). Data were normalized to PUMA2A (negative control) and alamethicin (positive control). The dynamic BH3 profile (ΔMOMP) was calculated by subtracting the percentage treated MOMP from percentage untreated MOMP.

Flow Cytometry-Based Apoptosis Assay

Cells were incubated at 0.1×10^6 cells/mL with AZD1775 (WEE1 inhibitor, Selleckchem) with or without QVD-Oph (pan-caspase inhibitor, Selleckchem, No. S7311) for 18, 24 or 72 h. After incubation, cells were washed with 1% BSA in PBS and stained with Annexin V-FITC (IQP-120F, IQ products, Groningen, The Netherlands) for 20 min on ice. Cells were washed with 1% BSA in PBS and stained with propidium iodide (Sigma Aldrich) to assess early apoptosis (Annexin V positive/propidium iodide negative) and late apoptosis (Annexin V positive/propidium iodide positive). Apoptosis was measured by flow cytometry (FACSCalibur, BD Biosciences). Data were analysed in Winlist 3D (Verity Software house, Topsham, ME, USA).

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% BSA/PBS and resuspended in solution containing 0.1% sodium citrate (A0158348, Merck, Kenilworth, New Jersey, USA), 0.01% propidium iodide (P4170, Sigma Aldrich), 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, Topsham, ME, USA).

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 Aldrich) according to the protocol provided with the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher, #00-5523-00, Waltham, MA, USA). Samples were processed on a MACSQuant and the data were analyzed using Kaluza 1.5 analysis software (Beckman, Brea, CA, USA).

Flow Cytometry-Based Viability Assay

Cells were incubated at 0.1×10^6 cells/mL with AZD1775 (WEE1 inhibitor, Selleckchem) for 18 h. After washing with 1% BSA in PBS, cells were incubated with venetoclax/ABT-199 (BCL-2-selective inhibitor, Selleckchem), S63845 (MCL-1 inhibitor, Selleckchem), or navitoclax/ABT-263 (BCL-XL/BCL-2/BCL-W inhibitor Selleckchem) for 48 h at 37 °C. After incubation, cells were washed with 1% BSA in PBS and stained with propidium iodide (Sigma Aldrich) to assess cell viability by flow cytometry (FACSCalibur, BD Biosciences). Data were analysed in Winlist 3D (Verity Software House, Topsham ME, USA).

Statistical Analysis

A one-sample T-test was used to assess the significance of the dynamic BH3 profiling results

($n=3$ independent repeats). Comparison of IC50 values between untreated and AZD1775-treated samples was done using a two-tailed paired T-test. Correlation analysis was carried out using Pearson's correlation test. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

Results

AZD1775 Induces Apoptosis and Enhances Anti-Apoptotic Dependency in DLBCL

DLBCL is a genomic unstable and heterogeneous cancer that shows a variety of molecular phenotypes. We previously demonstrated high expression of WEE1 in DLBCL cells compared to normal B-cells [4] and found this difference is significantly higher in DLBCL compared to other cancers (Supplemental figure 1). These data suggest that genomically unstable tumours such as DLBCL might be particularly sensitive to WEE1 inhibition. Thereto, we selected a panel of DLBCL cell lines with different genetic aberrations (Table S1). Treatment of DLBCL cell lines with WEE1 inhibitor AZD1775 induced a rapid decline in cell viability, with IC50 values ranging from 0.4 to 1.8 μM (Figure 1A and Supplemental table 1).

Cell death was induced by apoptosis, as measured by flow cytometry for annexin V/PI staining (Figure 1B). Apoptosis induced by AZD1775 could be observed in both a time and dose-dependent manner and could be rescued by pan-caspase inhibitor QVD (Supplemental figure 2). Next, we used dynamic BH3 profiling (Figure 1C and Supplemental table 2), which measures the changes in anti-apoptotic dependency upon treatment with AZD1775. As a result of AZD1775 treatment, cell lines OCI-LY3, SUHDL-6, SUDHL-10, and SC-1 had increased mitochondrial response to the pro-apoptotic peptide BIM at 27% ($p=0.0058$), 10% (ns), 14% ($p=0.0480$), and 8% (ns), respectively, indicating cells were more primed to undergo apoptosis. BH3 profiling with a mean $\Delta\text{MOMP} \geq 20\%$ was classified as biologically relevant, even if they were not statically significant, as they often lead to significant enhanced sensitivity to BH3 mimetic drugs, indicating biological relevance. In addition, the changes induced by AZD1775 treatment could be induced in a dose-dependent manner (Supplemental figure 3A) and were significantly correlated to the percentage of apoptotic cells (Supplemental figure 3B-C).

To investigate whether AZD1775-treated cells try to resist apoptosis, we next studied the dynamics of anti-apoptotic proteins MCL-1, BCL-XL, and BXL-2 in response to WEE1 inhibition as measured by mitochondrial response for NOXA, HRK, and BAD, respectively (Figure 1C). A significantly increased mitochondrial response to NOXA was observed in SUDHL-5 (12%, $p=0.0256$) and in SUDHL-10 (13%, $p=0.0393$), indicating increased dependency on MCL-1 upon AZD1775 treatment. For cell lines OCI-LY3 and SC-1, a significantly increased response was observed for HRK (21%; $p=0.0866$ and 27%; $p=0.0223$, respectively), demonstrating WEE1 inhibition increased dependency on BCL-XL. Most cell lines showed an increased mitochondrial response to BAD upon AZD1775 treatment, which reached 43%

Figure 1. Apoptosis induced by AZD1775 in diffuse large B-cell lymphoma (DLBCL). (A) Cell viability analysis was measured with propidium iodide flow cytometry in DLBCL cell lines treated with AZD1775 for 72 h. Data were plotted as the mean $\pm$ S.D. (n=3). (B) Representative flow cytometry of apoptosis (Annexin V/Propidium iodide) induced by treatment of AZD1775 for 18h in representative cell lines SUDHL-10 and SC-1. (C) Mitochondrial response of DLBCL cell lines treated with AZD1775 for 18h, plotted as the delta mitochondrial outer membrane permeabilization (Δ MOMP%). Δ MOMP% was calculated by subtracting the percentage treated MOMP from percentage untreated MOMP. Cell line SUDHL-10 was treated with 0.25 μ M AZD1775, cell lines OCI-LY3, U-2932, SUDHL-2, and SUDHL-5 were treated with 0.5 μ M AZD1775 and cell lines SUDHL-4, SUDHL-6, and SC-1 were treated with 1 μ M AZD1775. Data were plotted as the mean $\pm$ S.D. (n=3). Statistical analysis was performed using a one-sample T-test as compared to untreated control cells (* $p \leq 0.05$).

in OCI-LY3 ($p=0.0195$), 20% in U-2932 (ns), 23% in SUDHL-4 ($p=0.0317$), 11% in SUDHL-6 ($p=0.0223$), and 30% in SC-1 ($p=0.0540$). These results suggest that AZD1775 treatment leads to an increased dependency on BCL-2/BCL-XL/BCL-W. Only in cell line SUDHL-2, AZD1775 treatment did not induce changes in the anti-apoptotic dependency (Figure 1C), despite being relatively sensitive to AZD1775 treatment (Figure 1A). These results suggest that perhaps other apoptosis pathways, such as the extrinsic pathway, might be involved in the induction of apoptosis in SUDHL-2. Dynamic changes induced by AZD1775 in the different DLBCL cell lines showed no significant differences dependent on the GCB or ABC-subtype of DLBCL cell lines nor the TP53 status (Supplemental table 1). In conclusion, AZD1775 induces cell death through apoptosis, which enhanced the dependency on anti-apoptotic proteins.

DNA Damage and Premature Mitotic Entry Induced by AZD1775 Enhance Anti-Apoptotic Dependency

WEE1 inhibition by AZD1775 induces both premature mitotic entry [21,22] and DNA damage [23] in various types of cancer cells. To confirm that AZD1775 has a similar effect on DLBCL cells, we analysed cell cycle distribution and γ H2AX expression as a marker for DNA damage in the representative MCL-1 dependent cell line SUDHL-10 and the BCL-2 dependent cell line SC-1 (Figure 2A). AZD1775 treatment resulted in a dose-dependent increase in the percentage of G2/M cells, indicating premature mitotic entry and/or prolonged mitotic arrest and a dose-dependent increase of DNA damage (γ H2AX positive cells) in both SUDHL-10 and SC-1 (Figure 2A).

Figure 2. Cellular effect of AZD1775 treatment in DLBCL. (A) Representative examples of flow cytometry cell cycle and γ H2AX analysis of AZD1775 in SUDHL-10 and SC-1 treated for 18h. (B) Correlation of the mitochondrial response (Δ MOMP%) for 10 μ M NOXA in SUDHL-10 or 0.1 μ M BAD in SC-1 versus the percentage of G1 phase cells treated with AZD1775. (C) Correlation of the mitochondrial response (Δ MOMP%) for 10 μ M NOXA in SUDHL-10 or 0.1 μ M BAD in SC-1 versus the percentage of S/G2/M phase cells treated with AZD1775. (D) Correlation of the mitochondrial response (Δ MOMP%) of 10 μ M NOXA in SUDHL-10 or 0.1 μ M BAD in SC-1 versus the percentage of γ H2AX cells treated with AZD1775. Cells were treated with 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, or 1.0 μ M AZD1775 for 18 h. (E) Representative examples of cell cycle flow cytometry and γ H2AX analysis in SC-1 treated with 1 μ M AZD1775, 50 ng/mL nocodazole, and 20 J/m² UV radiation. (F) Dynamic BH3 profile for 0.3 μ M BIM, 10 μ M NOXA, 10 μ M HRK, and 0.1 μ M BAD of SC-1 cells treated with AZD1775, nocodazole, and (ultra violet) UV radiation. Data were plotted as the mean $\pm$ S.D. ($n=3$). Statistical analysis was performed using a one-sample T-test as compared to untreated control cells (* $p \leq 0.05$).

In addition, dynamic BH3 profiling showed a dose-dependent correlation between mitochondrial response to BAD in SC-1 cells treated with AZD1775 and the decrease in the percentage of G1 phase cells ($R=-0.8544$, $p=0.0069$) (Figure 2B), an increase in the percentage of S/G2/M phase cells ($R=0.8646$, $p=0.0056$) (Figure 2C), and an increase in γ H2AX levels ($R=0.8383$, $p=0.0093$) (Figure 2D). Similar correlation for response to NOXA and G-1 phase cells ($R=-0.7817$, $p=0.0219$), percentage of S/G2/M phase cells ($R=0.9036$, $p=0.0021$), and γ H2AX ($R=0.8822$, $p=0.0037$) were observed in SUDHL-10. These results indicate a potential relation between G2/M cell cycle arrest and increased DNA damage that could affect the anti-apoptotic dependency in DLBCL cells.

Next, we investigated if cell cycle distribution and/or DNA damage could induce changes in anti-apoptotic dependency, by exploring the ability of nocodazole to induce cell cycle arrest and of ultraviolet radiation (UV) to induce γ H2AX (Figure 2E-F and Supplemental figure 4) in SC-1 cells. Nocodazole treatment induced an increase in the percentage of cells in the G2/M phase and a moderate (6%) increase in γ H2AX-positive cells. UV exposure had a limited effect on the cell cycle distribution, but induced γ H2AX in about 60% of the cells (Figure 2E and Supplemental figure 4). Dynamic BH3 profiling revealed that both nocodazole and UV exposure induced a significant increase in the mitochondrial response to BIM, of 24% ($p=0.0249$) and 45% ($p=0.0224$), respectively (Figure 2F). Similarly, response levels to BAD significantly increased, at 39% ($p=0.0027$) and 46% ($p=0.0307$), respectively.

To further test the hypothesis that WEE1 inhibition induces changes in anti-apoptotic dependency as a result of cell cycle changes and DNA damage, we tested the combination of AZD1775 together with the CDK1 inhibitor RO3306, CDK4/6 inhibitor palbociclib, and CDK1/2 inhibitor roscovitine (Figure 3 and Supplemental figure 4), which has previously been shown to rescue AZD1775-induced DNA damage [24,25].

Treatment of SC-1 cells with the (single agent) roscovitine had little effects on either apoptosis levels, the cell cycle, or levels of DNA damage (Figure 3A,B). However, when combined with AZD1775, roscovitine significantly reduced DNA damage levels (from 75% to 7%, $p=0.0024$) and largely prevented alterations in cell cycle distribution (Figure 3A,B). Treatment with roscovitine alone or in combination with AZD1775 did not lead to an altered dynamic BH3 profile, demonstrating that a full rescue of both cell cycle distribution and DNA damage prevents anti-apoptotic changes induced by AZD1775 (Figure 3B). In addition, we tested CDK1 inhibitor RO3306 (Figure 3A,B and Supplemental figure 5), which rescues only AZD1775-induced DNA damage, and CDK4/6 inhibitor palbociclib (Figure 3A,B and Supplemental figure 5 and 6), which rescues only AZD1775-induced cell cycle arrest. Neither partial rescue of DNA damage (Supplemental figure 4) nor cell cycle distribution (Figure 3A) were sufficient to prevent anti-apoptotic changes induced by AZD1775. Based on these results, we conclude that both DNA damage and cell cycle disruption induced independently changes in the mitochondria and enhanced anti-apoptotic dependency.

Figure 3. Rescue of AZD1775-induced cellular effects by CDK inhibitors RO3306, palbociclib and roscovitine.

(A) Representative examples of apoptotic cells, cell cycle, and γ H2AX flow cytometry analysis of SC-1 treated with 10 μ M RO3306, 1 μ M palbociclib, 15 μ M roscovitine, and 1 μ M AZD1775 after 18 h of incubation. (B) Dynamic BH3 profile for 0.3 μ M BIM, 10 μ M NOXA, 10 μ M HRK, and 0.1 μ M BAD of SC-1 cells treated with RO3306, palbociclib, roscovitine, and AZD1775 after 18 h of incubation. Delta mitochondrial outer membrane permeabilization (Δ MOMP%) was calculated by subtracting the percentage treated MOMP from percentage untreated MOMP. Data were plotted as the mean $\pm$ S.D. ($n=3$). Statistical analysis was performed using a one-sample T-test as compared to untreated control cells (* $p \leq 0.05$).

AZD1775 Increases Sensitivity to BCL-2 and MCL-1 Inhibitors in DLBCL

Now that we have established that DNA damage and cell cycle disruption by AZD1775 (Figure 2) is sufficient to induce intrinsic apoptosis (Supplemental figures 1–3) and enhance dependency on anti-apoptotic proteins to prevent apoptosis, we next studied the combination of AZD1775 with anti-apoptotic inhibitors. We used SC-1 as a cell line with enhanced dependency on BCL-2/BCL-XL/BCL-W, and SUDHL-10 and SUDHL-5 as cell lines with enhanced dependency on MCL-1 as predicted by BH3 profiling (Figure 1C). Combined treatment of AZD1775 and venetoclax caused a 10-fold decrease in cell viability in SC-1 (Figure 4A).

A significant decline in viability from 82% to 26% ($p < 0.0001$) was observed when 0.01 μ M venetoclax was combined with 1 μ M AZD1775, resulting in an IC₅₀ decrease from 0.04 μ M in AZD1775-untreated cells to 0.003 μ M in AZD1775-treated cells for venetoclax ($p = 0.0039$, Figure 4B). In the MCL-1 dependent cell lines SUDHL-10 and SUDHL-5, as expected, co-treatment with AZD1775 did not enhance sensitivity to venetoclax. In these cell

lines, AZD1775 induced enhanced dependency on MCL-1, suggesting cells would become more sensitive to MCL-1 inhibitor S63845.

Figure 4. Combination of AZD1775 and BH3 mimetic drugs in DLBCL cell lines. (A) Cell viability flow cytometry data for the cell lines SC-1, SUDHL-5, and SUDHL-10 pre-treated with 1 μ M AZD1775 and incubated with increasing concentrations of venetoclax. Data were normalized to the control and plotted as the mean $\pm$ S.D. ($n=4$). (B) Venetoclax IC₅₀ values as calculated from cell viability curves. Data were plotted as the mean $\pm$ S.D. ($n=4$). (C) Cell viability flow cytometry data for SC-1, SUDHL-5, and SUDHL-10 pre-treated with 1 μ M AZD1775 and incubated with increasing concentrations of S63845. Data were normalized to the control and plotted as the mean $\pm$ S.D. ($n=4$). (D) S63845 IC₅₀ values as calculated from cell viability curves. Data plotted as the mean $\pm$ S.D. ($n=4$). Statistical analysis was performed using a two-tailed paired T-test (* $p \leq 0.05$) (** $p \leq 0.01$).

Combined treatment of 1 μ M AZD1775 and S63845 indeed caused a dose-dependent decrease in cell viability in the cell line SUDHL-5 (from 61% to 31% ($p=0.0024$) at 50 nM

S63845) and in cell line and SUDHL-10 (from 83% to 40% ($p=0.0003$) at 50 nM S63845) (Figure 4C). These changes resulted in a 7-fold decrease in IC₅₀ values for S63845 in SUDHL-10 (from 544 to 77 nM, $p=0.0658$) and a 2-fold decrease in IC₅₀ values for S63845 in SUDHL-5 (from 61 to 35 nM, $p=0.0028$) (Figure 4D). No effect on cell viability was observed when AZD1775 was combined with S63845 in SC-1 (Figure 1C). These results confirm that the changes observed in dynamic BH3 profiling upon AZD1775 treatment enhance dependency on anti-apoptotic proteins, a dependency that can be effectively exploited through targeting with the appropriate BH3 mimetic.

AZD1775 Alters the Anti-Apoptotic Dependency of Patient-Derived DLBCL Cells

Finally, we studied anti-apoptotic dependency and the dynamic BH3 profile following AZD1775 treatment of lymphoma cells from a DLBCL patient (Supplemental figure 7). Efficient apoptotic priming was observed (Figure 5A), and dynamic BH3 profiling (Figure 5B) showed an increase in the mitochondrial response to BAD (56% at 0.1 μ M BAD) but not to HRK after AZD1775 treatment, suggesting an increased sensitivity to BCL-2 or BCL-W inhibitors, but not to BCL-XL inhibitors.

Figure 5. Dynamic BH3 profile and validation experiments in a DLBCL patient treated with AZD1775. (A) Static BH3 profile and (B) dynamic BH3 profile of DLBCL patient cells treated with 0.5 μ M AZD1775 for 18 h. Alamesticin and PUMA2A were used as positive and negative controls, respectively. Delta mitochondrial outer membrane permeabilization (Δ MOMP%) was calculated by subtracting the percentage treated MOMP from percentage untreated MOMP. (C) Cell viability flow cytometry data for DLBCL patient cells pre-treated with AZD1775 for 18 h and incubated with increasing concentrations of venetoclax and (D) navitoclax for 24 h. Data were normalized to the control.

These findings were validated by *ex vivo* treatment of lymphoma cells with AZD1775 at increasing concentrations of venetoclax (BCL-2i) or navitoclax (BCL-2i, BCL-XL, and BCL-Wi). Venetoclax did not alter cell viability (Figure 5C), but navitoclax induced a 2-fold decrease

in cell viability (Figure 5D), together with a decrease in IC₅₀ from 0.015 μ M for navitoclax alone to 0.0067 μ M for navitoclax combined with AZD1775, indicating a dependency on BCL-W. We were unable to study the effect of BCL-W inhibition, as specific BCL-W inhibitors are not currently available. Taken together, these data emphasize the potential of AZD1775 combined with BH3 mimetics in the treatment of DLBCL patients and underscore the clinical utility of BH3 profiling.

Discussion

In genomically unstable cancers such as DLBCL, WEE1 is highly expressed and a relevant target for therapy. Since the WEE1 inhibitor AZD1775 is indeed effective in inducing apoptosis we investigated if it could alter anti-apoptotic dependency and increase sensitivity to BH3 mimetic drugs in DLBCL cell lines and patient material. Our findings demonstrate that [1] AZD1775 induces cell death through apoptosis; [2] AZD1775-mediated inhibition of WEE1 alters the anti-apoptotic dependency in DLBCL; [3] combination of AZD1775 with cell-specific anti-apoptotic inhibitors (such as venetoclax) leads to enhanced potency; [4] both DNA damage and G2/M arrest induced by WEE1 inhibition independently enhances dependency on anti-apoptotic proteins. Based on these results, we propose a model in which cell cycle disruption (such as premature mitotic entry or G2/M arrest) and DNA damage can induce changes in the mitochondrial response, resulting in an altered dependency on anti-apoptotic proteins in DLBCL cells (Figure 6).

Figure 6. Proposed mechanism for the relation between inhibition of cell cycle regulator WEE1 and the resistance of apoptosis. As a result of WEE1 inhibition cells are able to prematurely enter mitosis, without proper repair of genomic aberrations or mutations. This elimination of the checkpoint at the transition from the G2-phase into the M-phase leads to continuous cell cycling and results in high levels of DNA damage that activate apoptosis pathways. To prevent apoptosis, cancerous cells become increasingly dependent on anti-apoptotic proteins such as BCL-2, MCL-1, BCL-XL, and BCL-W, which ensure survival under high levels of genomic stress.

In normal B-cell development, dependency on anti-apoptotic proteins changes during B-cell maturation. Naïve B-cells are dependent on BCL-2, GC B-cells shift to dependency on MCL-1, memory B-cells are once more dependent on BCL-2, while plasma cells depend

on BCL-XL [26]. The t(14;18) IGH-BCL-2 translocation, found in ~20% of DLBCL patients, is a hallmark of follicular lymphoma and is more commonly found in the germinal centre derived-DLBCL subtype [14,27]. BCL-W has a potentially important role in B-cell survival, as overexpression of the BCL-W gene is associated with a worse prognosis in specific DLBCL cases [28]. Using baseline BH3 profiles and sensitivity to BH3 mimetic drugs, we showed that DLBCL cell lines are dependent on multiple anti-apoptotic proteins. These associations showed no direct relationship to the cell of origin, thus highlighting the heterogeneity of DLBCL.

To date, the primary application of dynamic BH3 profiling has been measurement of responses to chemotherapy [17]. Here, we were able to accurately predict changes in anti-apoptotic dependency profiles as a result of WEE1 inhibition by AZD1775. These dependencies changed both quantitatively and qualitatively. For example, dynamic BH3 profiling highlighted interesting shifts in dependency, such as the shift to dependency on BCL-XL after AZD1775 treatment of the previously BCL-XL independent SC1 cell line. This shows that tumour cells can shift their dependency from one anti-apoptotic protein to another. Unfortunately, current multiple anti-apoptotic protein inhibitors, including those targeting BCL-XL such as navitoclax, are poorly tolerated and have significant side effects [29]. Inhibition of BCL-XL also shortens platelet lifespan [30], hampering use of these agents. Nevertheless, as tumour cells appear able to use and adapt their dependency to multiple anti-apoptotic proteins, there is clearly a need for new, tolerated multi-target inhibitors in order to prevent resistance and relapse in DLBCL patients. Our results demonstrate the broad range of effects of AZD1775 on cellular states, including the ability to influence apoptotic pathways, clearly highlighting potential therapeutic targets. It can be argued that analyses of protein levels by western blot or immunohistochemistry, perhaps including RNA expression levels, are suboptimal approaches when determining the anti-apoptotic dependency of tumour cells. By contrast, static BH3 profiling of cell lines or primary patient samples has proven itself as a fast and reliable tool to establish the functionality of and dependency on anti-apoptotic proteins.

Treatment of solid cancers [11] and leukaemia [12,13] with inhibitors of the cell cycle regulator WEE1 has proven successful in early clinical trials, as WEE1 inhibitors result in cell cycle disruption [21,22], induction of DNA damage [23,31] and eventually, induction of apoptosis. Our data highlighted a novel effect of WEE1 inhibition, an altered dependency on anti-apoptotic proteins. Rescue experiments preventing either DNA damage and/or G2/M cell cycle arrest after WEE1 inhibition showed that both events are able to induce changes in anti-apoptotic dependency, either individually or in combination. Mitochondrial fission at mitosis is known to be tightly regulated by the CDK1-cyclinB complex, which phosphorylates the GTPase Drp1 and thus induces mitochondrial fragmentation to promote mitochondrial fission [32,33]. Loss of Drp1 results in dysfunctional mitochondrial fission, persistent mitochondrial hyperfusion, delayed G2/M cell cycle progression, replication stress, DNA damage, ATM activation, and genomic instability [34]. Since WEE1 is the main regulator of the CDK1-cyclinB complex, loss of WEE1 will induce premature mitochondrial fragmentation, destabilizing the connection between cell cycle division and mitochondrial homeostasis. Similarly, activation of the DNA damage response protein ATM in response to double-

stranded DNA breaks results in phosphorylation of the BH3 activator protein BID, initiating DNA damage-induced apoptosis [35]. Here, we show a novel direct link between the cell cycle, DNA damage and mitochondrial apoptosis, in which either premature mitotic entry or DNA damage induced by WEE1 inhibition alters anti-apoptotic dependency. Although this mechanism was not tested in other cancer types, based on the fundamental role of WEE1 in cell cycle regulation we expect a similar association and response in other cell types. With the current development of BH3 mimetics, WEE1 inhibition would be an excellent candidate for dose reduction combination therapy in treatment-naïve patients, or could be applied as a BH3 mimetic sensitizer in WEE1 inhibitor-resistant tumours.

In conclusion, we demonstrated for the first time that WEE1 inhibition in DLBCL can lead to further sensitization to anti-apoptotic inhibitors, revealing a novel mechanism and as yet unexplored application for WEE1 inhibition. Using cell cycle and DNA damage rescue experiments, we unraveled the mechanism underlying changes in anti-apoptotic dependency, highlighting mechanisms that may have applications in the treatment of other cancers. These findings suggest important applications for WEE1 as a novel therapeutic treatment approach, but also suggest broader possibilities for genotoxic drugs, cell cycle deregulators, or DNA damage response inhibitors in combination with BH3 mimetic drugs. Finally, we showed that combining WEE1 inhibition with dynamic BH3 profiling represents an educated approach to guided therapy, and may lead to novel strategies in optimized/personalized treatment selection in DLBCL patients.

Conclusions

In conclusion, we demonstrate that treatment of cells with WEE1 inhibitor AZD1775 can enhance the dependency on anti-apoptotic proteins and enhanced sensitivity to BH3 mimetic drugs. In addition, we showed that DNA damage and cell cycle arrest independently induce similar changes in the anti-apoptotic dependency. Therefore, we predict that other DNA damage-inducing or cell cycle arresting agents will synergize with BH3 mimetic drugs.

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Supporting information

Supplemental Table 1. Characteristics of DLBCL cell lines. Characteristics of the DLBCL cell lines and their cell of origin (COO), IC50 value for AZD1775, TP53 mutation status and cytogenetic profile (DSMZ/ATCC).

Cell line	COO subtype	AZD1775 IC50 (μM)	TP53 status	TP53 mutation	Cytogenetics (DSMZ or ATCC)
OCI-LY3	ABC	0.68	WT	x	Human flat-moded hypertriploid karyotype; 72-77<3n>XXYY, +1, +9, -10, +13, +14, -17, +19, +20, +22, der(1)t(1;17)(p13;q12)x2, der(4)t(4;18)(q31;q21)x2, del(6)(q13)x2, der(6)t(6;6)(p24;q12), der(7)t(6;7)(p24;p22), der(14)t(14;19)(q32;q13.3)x2, del(18)(q21), der(19)t(4;19)(q21;q13)t(4;18)(q31;q21)x2, der(19)t(14;19), dup(20)(q11q13)x2; sdi with der(6)t(6;12)(p21;q21), der(7)t(5;7)(?p15;p24) etc; resembles published karyotypes; carries cryptic t(14;19) with rearrangement of IGH and SPIB, and t(4;18) with copy number amplification of the BCL2 region.
U-2932	ABC	1.02	MUT	c.527G>A	Human polyclonal hypodiploid karyotype with 20% polyploidy; 45(43-46)<2n>, XX, add(X)(q22), add(1)(q24), der(3)ins(3;18)(q27;q27q27)hsr(18)(q21), add(5)(q32), der(6)t(6;18)(p24;q23)del(6)(q13), der(10)t(10;14)(q24;q23), der(11)t(1;11)(q25;q27), der(14)t(3;14)(q27;p11), der(18)t(1;18)(q21;q21), der(18)t(3;18)(q27;q27), add(19)(p13), del(19)(q13); sdi with del(2)(q11), t(4;15)(q22;q14), add(7)(q21), del(13)(?q21), der(13)add(13)(p11)add(13)(q32); extensive genomic amplification of BCL2 region; resembles published karyotype.
SUDHL-2	ABC	0.42	MUT	c.701A>C	Most of these cells were hyperdiploid, with a sharp modal number of 51 chromosomes; occasional cells in the hypertetraploid range were also seen. A minute marker chromosome was consistently observed, and increased numbers of chromosomes were seen in the A, B, C, and F groups.
SUDHL-4	GCB	0.99	MUT	c.817C>T	Human hyperdiploid karyotype with 4% polyploidy - 50(47-51)<2n>XY/XXY, -3, +7, +8, +12, +13, -16, +18, der(3)t(3;3)(p27;q12), der(8)t(3;8)(p12;q12)inv(3)(p12;p27), der(12)t(3;12)(p12;q24)add(3)(p27), add(13)(p11), t(14;18)(q32.3;q21.1), add(16)(p13), der(18)t(14;18)(q32.3;q21.1) - carries t(14;18) effecting BCL2(MBR)-IGH fusion - matches published karyotype.
SUDHL-5	GCB	0.81	WT	x	Human hyperdiploid karyotype with 1.5% polyploidy - 47(41-48)<2n>XX, +12, del(6)(q13), del(12)(q13) - sideline with del(6)x2 - matches published karyotype.
SUDHL-6	GCB	1.42	MUT	c.701A>G	Human hyperdiploid karyotype with 2% polyploidy - 47(42-48)<2n>X, -Y, +6, +7, del(4)(q23), del(6)(p21.3p22.2), i(6p), der(7)(q22q32), der(8)t(8;9)(q24;p13), der(9)t(8;19)(q24;q13;p13), dup(11)(q24q25), t(14;18)(q32;q21), der(22)t(7;22)(?q32;p11) - sideline with dic(8;9)(q24;p13), ider(8)(q10)t(8;9)(q24;p13) - matches published karyotype - carries t(14;18) effecting IGH-BCL2 fusion.

Supplemental Table 1 continued.

Cell line	COO subtype	AZD1775 IC50 (μ M)	TP53 status	TP53 mutation	Cytogenetics (DSMZ or ATCC)
SUDHL-10	GCB	0.79	MUT	c . 9 9 4 - 1G>C	Human flat-moded hyperdiploid karyotype - 47(43-48)<2n>XY, +7, der(8)t(X;8)(q25;p23) t(8;X)(q24;q26)t(X;14)(q28;q32), del(10)(q22q24), der(11)t(Y;11)(q11;q25), der(14)t(8;14)(q24;q32), der(18)t(14;18)(q32;q21) - carries concurrent rearrangements of IGH with MYC and BCL2 - resembles published karyotype.
SC-1	GCB	0.83	WT	x	Human hyperdiploid karyotype with 4% polyploidy - 47(43-48)<2n>XY, +7, -17, +mar, add(3)(q27), t(8;14;18)(q24;q32;q21), add(14)(q32) - carries 3-way t(8,14,18) effecting genomic co-amplification of BCL2 and MYC - resembles published karyotype.

Supplemental Table 2. BH3 treatment schedule for DLBCL cell lines. Schematic overview of the treatment schedule applied to the DLBCL cell lines in order to establish the effect of AZD1775 on anti-apoptotic dependency via dynamic BH3 profiling.

Cell line	AZD1775 (μ M)	BIM (μ M)	NOXA (μ M)	HRK (μ M)	BAD (μ M)
OCI-LY3	0.5	0.1	10	10	0.1
U-2932	0.5	0.1	10	10	0.1
SUDHL-2	0.5	0.1	10	10	10
SUDHL-4	1	0.1	10	10	0.1
SUDHL-5	0.5	0.1	10	10	10
SUDHL-6	1	0.03	10	10	1
SUDHL-10	0.25	0.1	10	10	10
SC-1	1	0.3	10	10	0.1

Supplemental Figure 1. Gene expression levels of WEE1 in multiple cancer types. Plotted is the factor of tumor tissue / representative normal tissue gene expression for WEE1 across multiple types of cancers. Cancers included are adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), cholangio carcinoma (CHOL), colon adenocarcinoma (COAD), diffuse large B-cell lymphoma (DLBCL), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), acute myeloid leukemia (AML), brain lower grade Glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), mesothelioma (MESO), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumors (TGCT), thyroid carcinoma (THCA), Thymoma (THYM), uterine corpus endometrial Carcinoma (UCEC), uterine carcinosarcoma (UCS) and uveal melanoma (UVM). Data was acquired from the online available GEPIA database (gepia.cancer-pku.cn).

Supplemental Figure 2. Time and dose-dependent induction of apoptosis by AZD1775 in DLBCL. Flow cytometry analysis of early apoptosis (Annexin V positive/Propidium iodide negative) and late apoptosis (Annexin V positive/Propidium iodide positive) in cell lines SUDHL-10, SC-1 and U-2932 treated with AZD1775 $\pm$ 20 μ M caspase inhibitor QVD for 18, 24 or 48 h. Plotted is the mean of $n=3$.

Supplemental Figure 3. Dose-response effect of AZD1775 on mitochondrial outer membrane permeabilization. (A) Dynamic BH3 profile of cell lines SUDHL-10 and SC-1 cell line treated with an increasing concentration of AZD1775 for 18 h. Delta mitochondrial outer membrane permeabilization (ΔMOMP%) for 10 μM NOXA for cell line SUDHL-10 and 0.1 μM BAD for cell line SC-1 was calculated by subtracting the percentage treated MOMP from percentage untreated MOMP. Data were plotted as the mean ± S.D. (n=3). Statistical analysis was performed using a one-sample T-test as compared to untreated control cells (*p≤0.05). (B) Flow cytometry analysis of early apoptosis (Annexin V positive/Propidium iodide negative) and late apoptosis (Annexin V positive/Propidium iodide positive) in cell lines SUDHL-10 and SC-1 treated with AZD1775 ± 20 μM caspase inhibitor QVD for 18 h. Plotted is the mean of n=3. (C) Correlation of the ΔMOMP% for 10 μM NOXA in SUDHL-10 and 0.1 μM BAD in cell line SC-1 and the percentage of early apoptotic cells (Annexin V positive/Propidium iodide negative) after 18 h of AZD1775 treatment.

Supplemental Figure 4. DNA damage induced by AZD1775, nocodazole or UV treatment. Percentage of γH2AX positive cells after treatment of cell line SC-1 with 1 μM AZD1775, 50ng/mL nocodazole or 20 J/m2 after 18 hours of incubation. Data were plotted as the mean ± S.D. (n=3). Statistical analysis was performed using a two-tailed paired T-test comparing treatment to control (*p≤0.05) (**p≤0.01).

Supplemental Figure 5. DNA damage induced by AZD1775 alone or combined with CDK inhibitors. Percentage of γH2AX positive cells after treatment of cell line SC-1 with 10 μM RO3306, 1 μM palbociclib, 15 μM roscovitine and 1 μM AZD1775 after 18 h of incubation. Data were plotted as the mean ± S.D. (n=3). Statistical analysis was performed using a two-tailed paired T-test comparing treatment to control (*p≤0.05) (**p≤0.01).

Supplemental Figure 6. Correlation of dynamic BAD BH3 profile and γH2AX after Palbociclib and AZD1775. (A) Correlation of the (ΔMOMP%) for 0.1 μM BAD versus the percentage of γH2AX cells in cell line SC-1 cells treated with AZD1775 and palbociclib for 18 h.

Supplemental Figure 7. DLBCL patient characteristics. (A) Flow cytometry analysis of bone marrow aspirate. (B) Smear of bone marrow aspirate and (C) enlarged image of bone marrow aspirate smear. Depicted DLBCL patient sample contains 60% CD10+/CD19+/CD45+ monoclonal lymphoid B-cells.

Heterogeneous pattern of dependence on anti-apoptotic BCL-2 family proteins upon CHOP treatment in diffuse large B-cell lymphoma

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Abstract

Expression of the anti-apoptotic B-cell lymphoma 2 (BCL-2) protein in patients with diffuse large B-cell lymphoma (DLBCL) strongly correlates with resistance to standard therapy with cyclophosphamide, vincristine, doxorubicin, prednisolone, and rituximab (R-CHOP). Although studies focus mainly on the contribution of BCL-2, here we also investigate the contribution of other anti-apoptotic proteins to CHOP-therapy resistance in DLBCL. Functional dynamic BCL-2 homology (BH)3 profiling was applied to DLBCL cell lines upon CHOP treatment or single CHOP compounds. Cell-specific anti-apoptotic dependencies were validated with corresponding BH3-mimetics. We found high expression of anti-apoptotic BCL-2, MCL-1, and BCL-XL in DLBCL cell lines and patients. CHOP treatment resulted in both enhanced and altered anti-apoptotic dependency. Enhanced sensitivity to different BH3-mimetics after CHOP treatment was confirmed in specific cell lines, indicating heterogeneity of CHOP-induced resistance in DLBCL. Analysis of single CHOP compounds demonstrated that similar changes could also be induced by doxorubicin or vincristine, providing evidence for clinical combination therapies of doxorubicin or vincristine with BH3-mimetics in DLBCL. In conclusion, we show for the first time that CHOP treatment induces increased anti-apoptotic dependency on MCL-1 and BCL-XL, and not just BCL-2. These results provide new perspectives for the treatment of CHOP-resistant DLBCL and underline the potential of BH3 profiling in predicting therapy outcomes.

Introduction

The B-cell lymphoma 2 (BCL-2) family of proteins is a group of pro- and anti-apoptotic proteins with key roles in the regulation of intrinsic apoptosis. Proteins of the BCL-2 family share a general structure of one or more BCL-2 homology (BH) domains, of which expression of the BH3 domain is a universally shared feature. Expression of this BH3 domain is essential for protein-protein interactions, thereby allowing dimerization of pro-apoptotic sensitizer proteins (e.g., BAD, NOXA and HRK) with anti-apoptotic proteins (e.g., BCL-2, BCL-XL, MCL-1 and BCL-W). This interaction leaves the pro-apoptotic activator proteins (e.g., BID and BIM) free to interact with the effector proteins (e.g., BAX and BAK) to induce pore formation and cytochrome c release from the mitochondria, thus promoting apoptosis [1]. Dysregulated expression of several important anti-apoptotic proteins, such as BCL-2, plays a crucial role in carcinogenesis and anti-cancer therapy resistance [2]. The specific anti-apoptotic dependency in a particular cancer cell is often unknown, and cancer cells may adapt their anti-apoptotic dependencies when exposed to particular apoptosis-inducing therapies (e.g., chemotherapy) to further facilitate therapy resistance [3].

In diffuse large B-cell lymphoma (DLBCL), overexpression of the anti-apoptotic protein BCL-2 is correlated with adverse survival in patients treated with standard rituximab, cyclophosphamide, vincristine, doxorubicin, and prednisone (R-CHOP) [4]. In DLBCL patients, BCL-2 is commonly overexpressed due to either a t(14;18) chromosomal translocation or to BCL-2 amplification or transcriptional upregulation. The t(14;18) translocation is more common, at 20%–30% of cases, in the germinal center (GCB) molecular subtype, whereas BCL-2 amplification is found in 8%–30% of the activated B-cell (ABC) subtype [5,6].

Inhibition of the anti-apoptotic activity of BCL-2 has been suggested as a promising strategy for treatment [7], however, a single-agent phase I study of the specific BCL-2 inhibitor venetoclax in relapsed or refractory DLBCL reported limited efficacy [8]. On the other hand, promising results, including an overall response of 87.5% and a complete response (CR) of 79.2%, were reported in a phase I trial of venetoclax combined with R-CHOP as first-line treatment in non-Hodgkin lymphomas, including DLBCL [9]. However, other studies have reported that apoptotic resistance in DLBCL can be acquired through proteins other than BCL-2 [10]. Analysis of primary DLBCL patient samples treated with BH3 mimetic drugs (BCL-2 inhibitor and MCL-1 inhibitor) showed that patients could be subdivided into BCL-2-sensitive or MCL-1-sensitive subgroups [10]. However, the effect of standard (prolonged) CHOP treatment itself on DLBCL-specific pro- and anti-apoptotic signaling is currently unknown. To this end, we studied the effect of CHOP therapy on anti-apoptotic dependency in DLBCL using functional dynamic BH3 profiling [11]. By employing functional dynamic BH3 profiling, we compared the profiles of eight DLBCL cell lines before and after CHOP treatment and thus determined the change in dependency on anti-apoptotic proteins. Our results showed that CHOP resistance is not exclusively mediated through BCL-2, but by multiple other anti-apoptotic proteins. These results highlight the heterogeneity of anti-apoptotic dependency in DLBCL, which likely depends on other anti-apoptotic proteins in

addition to BCL-2. These results could have implications for clinical trials evaluating efficacy of BH3 mimetics.

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% fetal 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. Cell lines were obtained from ATCC and DSMZ. The identity of our cell lines was checked on a regular basis. Cell of origin (COO), BCL-2, and MYC status was based on data from ATCC and DSMZ.

Patient Material

Patient material was used from 55 DLBCL patients with primary DLBCL, including most subtypes. Material was acquired in accordance with international regulations and professional guidelines (the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice). Material used in this project (RR#201800551, 1 November 2018) was obtained from anonymous rest material. The medical ethics review board (Central Ethics Review Board non-WMO studies, University Medical Center Groningen (UMCG)) waives the need for approval if rest material is used, under the law in the Netherlands and waives the need for informed consent when patient anonymity is assured.

Immunohistochemistry (IHC)

IHC was performed with antibodies against BCL-2 (M0887, Dako, Glostrup, Denmark), BCL-XL (SC7195, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and MCL-1 (A3534, Dako, Glostrup, Denmark) on paraffin-embedded tissue sections after antigen retrieval (pH 6, pH 9, and pH 6, respectively). Staining was visualized using HRP-labeled secondary antibodies (Dako) and 3,3'-diaminobenzidine (Sigma-Aldrich, St Louis, MO, USA). Appropriate positive and negative controls were performed for each staining. The cases were stained and scored on a tissue micro-array (TMA). In the TMA, each case was represented by three tissue cores.

Compounds

The BCL-2 inhibitor venetoclax (#S8048, Houston, TX, USA), the BCL-2/BCL-XL/BCL-W inhibitor navitoclax (#S1001), and the MCL-1 inhibitor S63845 (#S8383) were all acquired from Selleckchem. CHOP chemotherapy consisted of cyclophosphamide (University Medical Center Groningen (UMCG) pharmacy, Groningen, the Netherlands), doxorubicin (#S1208, Selleckchem, Houston, TX, USA), vincristine (UMCG pharmacy, Groningen, the Netherlands), and prednisolone (#S1737, Selleckchem), in a composition set at the clinical ratio of 83/5.5/0.16/11.1, respectively [12].

BH3 Profiling-Plate-Based Assay

BH3 profiling is a method to functionally characterize the interplay between anti- and pro-apoptotic proteins for a particular cell line by employing a panel of pro-apoptotic BH3 domain peptides to measure specific anti-apoptotic protein reactions. Upon treatment with these peptides, the mitochondrial outer membrane response can be measured by cytochrome c release from the mitochondria using flow cytometry or as the altered mitochondrial membrane potential by measuring fluorescence emission of the JC-1 dye in a plate-based assay [3]. The BIM peptide is a pro-apoptotic activator protein that reacts with multiple anti-apoptotic BH-3 proteins and can be used to establish whether cells are able to undergo intrinsic apoptosis [11]. To determine the specific anti-apoptotic dependency of a DLBCL cell line, we looked at the mitochondrial response of cells to the pro-apoptotic sensitizers BAD, HRK, NOXA, or MS1. Cells dependent on anti-apoptotic BCL-2 will release cytochrome c in response to the BAD peptide, which interacts with BCL-2, BCL-XL, and BCL-W, but not HRK (which interacts only with BCL-XL). If cells are dependent on BCL-XL, cytochrome c release will be observed in response to both BAD and HRK peptides. Cells dependent on anti-apoptotic MCL-1 will show a response to NOXA and MS1. In order to establish the effect of CHOP with BH3, profiling cells were incubated at a density of 1.0×10^6 cells/mL for 18 h with CHOP. After incubation, cells were washed in mannitol experimental buffer (150 mM D-mannitol (M9647 Sigma-Aldrich, 3050 Spruce St., St. Louis, MO, USA), 10 mM HEPES (H3375 Sigma-Aldrich), 50 mM KCl (1.04936 Merck KGaA, Darmstadt, Germany), 20 nM EGTA (E4378 Sigma-Aldrich), 20 nM EDTA (11280, Serva Electrophoresis GmbH, Carl-Benz-Str. 7, Heidelberg, Germany), 0.1% bovine serum albumin (BSA) (11930 Serva Electrophoresis GmbH), 5 mM succinate (1.00682 Merck KGaA) dissolved in dH₂O, pH 7.5 (MEB)) and resuspended at 3.2×10^6 cells/mL in MEB. The cell suspension was mixed 1:1 with 4 μ M JC-1 permeabilization/staining solution (ENZ-52304, Enzo Life Sciences, 10 Executive Blvd., Farmingdale, NY 11735, USA), 0.004% digitonin (1500 643, Boehringer Mannheim GmbH, Mannheim, Germany), 20 mM β -mercaptoethanol (8.05740 Merck KGaA), and 40 μ g/mL oligomycin (O4876, Sigma-Aldrich) prepared in MEB) and incubated at room temperature for 10 min in the dark. BIM, PUMA, BAD, NOXA, HRK, BMF, and PUMA2A (JPT Peptide Technologies GmbH, Volmerstrasse, Berlin, Germany) were prepared in MEB in a black flat-

bottom non-treated polystyrene 96-well plate (3915 Costar, Corning Incorporated, 2 Alfred Road, Kennebunk, ME, USA). Peptide sequences used for the assay were identical to those described by Ryan and Letai ([13]). Plates were either used directly or sealed (Silverseal sealer, ref 676090, Greiner-Bio-One GmbH, Mayback Str., Frickenhausen, Germany), frozen at -80°C and thawed for one hour at room temperature before use. Cells in permeabilization/staining solution were added to the plate 1:1 at a final volume of $100\ \mu\text{L}$ and shaken for 15 s, followed by measurement of fluorescence (excitation $545\ \text{nm}$, emission $590\ \text{nm}$) every 5 min for 2 h at 30°C (Varioskan). All experiments were performed in triplicate or quadruplicate. Area under the curve (AUC) was calculated as the percentage mitochondrial outer membrane permeabilization (MOMP) and normalized to PUMA2A (negative control) and FCCP (positive control) with the formula:

$$1 - ((\text{AUC sample} - \text{AUC FCCP}) \div (\text{AUC PUMA2A} - \text{AUC FCCP})) \times 100\% \quad (1)$$

The dynamic BH3 profile (ΔMOMP) was calculated by subtracting the percentage treated MOMP from the percentage untreated MOMP. BH3 profiling with a mean $\Delta\text{MOMP} \geq 20\%$ were classified as biologically relevant, even if they were not always statically significant, as they often lead to significant enhanced sensitivity to BH3 mimetic drugs, indicating biological relevance.

Flow Cytometry—Cell Viability for IC50

For cell viability analysis, 0.2×10^6 cells/mL were treated for the appropriate times, washed with 1% BSA/PBS and resuspended in propidium iodide solution (P4170, Sigma). Samples were processed on a BD FACSCalibur 2 and analyzed with ModFit LT (Verity Software House, version 4.1.7, Topsham, ME, USA). Experiments were performed three times.

Resazurin—Metabolic Viability for Combination Therapies

After treatment of 0.4×10^6 cells/mL, 1/20th of the total volume of resazurin (AlamarBlue, Thermo Fisher Scientific, Waltham, MA, USA) was incubated for nine hours prior to read-out (Varioskan, excitation $560\ \text{nm}$, emission $590\ \text{nm}$). Experiments were performed 3 times.

Western Blot

Western blot was performed as previously described ([14]). Primary antibodies used were anti-BIM (1:1000, #2933 (C34C5) Cell Signaling Technology, Danvers, MA, USA), anti-MCL-1 (1:1000, #5453 (D35A5) Cell Signaling Technology), anti-BCL-2 (1:1000, ab32124 (E17), Abcam, Cambridge, United Kingdom), and anti-BCL-XL (1:1000, #2764 (54H6) Cell Signaling Technology).

Statistical Analysis

Data were analyzed using Graphpad PRISM (version 5.0, GraphPad Software, San Diego, CA, USA) software and tested for significant differences with a paired T-test. Correlation was analyzed using Spearman's correlation. Combination index (CI) was calculated using the Chou-Talalay method and CompuSyn software (ComboSyn Inc, NJ, USA). * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

Results

DLBCL Cells are Dependent on BCL-2 or MCL-1, but Not BCL-XL

BCL-2 protein overexpression is an important predictive and prognostic marker in DLBCL patients [4]. We first validated the importance of different anti-apoptotic BCL-2 family protein expression in our representative DLBCL cell line panel with different genetic backgrounds (5 GCB and 3 ABC) and correlated anti-apoptotic protein expression to their functional anti-apoptotic dependency as determined by BH3 profiling (Figures 1, S1, and S2).

All DLBCL cell lines showed protein expression of apoptotic activator BIM, indicating that cells are capable of undergoing apoptosis (Figure 1B). Variable protein expression of anti-apoptotic proteins MCL-1, BCL-XL, and BCL-2 was observed in the DLBCL cell lines, indicating that cells employed different and/or multiple anti-apoptotic proteins to protect from apoptosis. In most cases, cells with acquired translocation or amplification of the BCL-2 protein showed high expression of BCL-2, with the exception of cell line SUDHL-10, which showed high expression of MCL-1 instead (Figure 1A–B). Next, we performed BH3 profiling to determine the intrinsic functional dependency of cells on specific anti-apoptotic proteins (Figures 1C and Supplemental figure 1). BH3 profiling revealed that all cells showed a strong response to the BIM peptide (min 71%; max 95%), which confirms previous results for BIM protein expression (Figure 1B). In addition, cell lines with high BCL-2 protein expression showed a mitochondrial response to the BAD peptide (min 30%; max 93%), indicating functional dependency on BCL-2. Cell lines SUDHL-5 and SUDHL-10, which were not dependent on BCL-2, instead showed high response to the NOXA/MS1 peptides (min 17%; max 68%), indicating functional MCL-1 dependency, which matches with relatively high MCL-1 protein expression (Figure 1B). Together, these data demonstrate that DLBCL cells were either exclusively dependent on BCL-2 or MCL-1, but not on BCL-XL or multiple anti-apoptotic proteins simultaneously, despite expression of multiple anti-apoptotic proteins.

Figure 1. Expression and dependency on anti-apoptotic proteins in diffuse large B-cell lymphoma (DLBCL) cell lines. Overview of eight DLBCL cell lines with their (A) cell of origin (COO) classification, and translocation (TRANS) or amplification (AMP) status for B-cell lymphoma 2 (BCL-2) or MYC. (B) Protein expression of anti-apoptotic proteins encoded by BIM, MCL-1, BCL-XL and BCL-2. (C) BCL-2 homology (BH)3 profile peptide response as measured by mitochondrial outer membrane permeabilization (MOMP) ($n=3$). Values marked in red were below 10% MOMP and therefore classified as unresponsive, whereas values marked in green were above 10% MOMP and classified as responsive. (D) The half maximal inhibitory concentration (IC50) values for venetoclax (BCL-2i; 48 h), navitoclax (BCL-2/XL/Wi; 48 h), S63845 (MCL-1i; 48 h) and cyclophosphamide, vincristine, doxorubicin, prednisolone (CHOP) chemotherapy (72 h) ($n=3$). IC50 values below 1 μ M (for venetoclax, navitoclax, or S63845) or below 5 μ g/mL (for CHOP) were deemed sensitive and marked in red, whereas values above 1 μ M (for venetoclax, navitoclax, or S63845) or above 5 μ g/mL (for CHOP) were deemed insensitive and therefore marked in green.

DLBCL Patients Show Simultaneous Expression of BCL-2, BCL-XL, and MCL-1

To validate that, like the DLBCL cell lines, DLBCL patients also show simultaneous expression of BCL-2, BCL-XL, and MCL-1, we performed immunohistochemistry staining on 55 DLBCL patient tissues (Table 1 and Figure 2).

Staining for the anti-apoptotic proteins revealed 34/55 patients (62%) stained positive for BCL-2, 55/55 (100%) stained positive for BCL-XL, and 52/55 (95%) stained positive for MCL-1. In addition, one case (2%) was positive for only one anti-apoptotic protein (BCL-XL), 22 cases (40%) were positive for two out of three anti-apoptotic proteins, and 32 cases (58%) were positive for all three anti-apoptotic proteins. Of the BCL-2 negative cases, 21/21 (100%) were positive for BCL-XL and 20/21 (95%) were positive for MCL-1 (Table 1 and Figure 2A–C). A similar trend was observed in the BCL-2 positive cases, of which 33/34 (97%) stained positive for BCL-XL and 32/34 (94%) were positive for MCL-1 (Table 1 and Figure 2D–F). Together, these data demonstrate that there are high levels and simultaneous expression of the multiple anti-apoptotic proteins, which strongly mirror our findings in the DLBCL cell lines.

Table 1. Immunohistochemistry of BCL-2, BCL-XL and MCL-1 in DLBCL patients (n = 55).

DLBCL cases	BCL-2 Negative (n = 21)	BCL-2 Positive (n = 34)
BCL-XL negative	0/21 (0%)	1/34 (3%)
BCL-XL positive	21/21 (100%)	33/34 (97%)
MCL-1 negative	1/21 (5%)	2/34 (6%)
MCL-1 positive	20/21 (95%)	32/34 (94%)

Figure 2. Immunohistochemistry staining for BCL-2, MCL-1, and BCL-XL in DLBCL. (A) Representative example of a BCL-2 negative DLBCL patient with positive staining for MCL-1 (B) and BCL-XL (C). (D) Representative example of a BCL-2 positive DLBCL patient with positive staining for MCL-1 (E) and BCL-XL (F). All images were captured at 200x magnification.

BCL-2 Expression Predicts Sensitivity to Venetoclax and CHOP

Next, we correlated the intrinsic functional anti-apoptotic dependency of our cells to the sensitivity for anti-apoptotic inhibitors venetoclax (BCL-2i), S63845 (MCL-1i), navitoclax (BCL-2/BCL-XL/BCL-Wi), and CHOP chemotherapy (Figure 3).

We found a negative correlation between venetoclax IC50 and the BAD response ($R=-0.810$; $p=0.022$) (Figure 3A), but not for navitoclax and BAD response ($R=-0.575$; $p=0.143$) (Figure 3B), indicating that a high BAD response is predictive of a low venetoclax IC50 and thus high sensitivity to BCL-2 inhibition. This discrepancy between venetoclax and navitoclax is likely caused by the individual potency of the inhibitors, as venetoclax has a very high sensitivity for BCL-2 ($K_i < 0.01$ nM) compared to navitoclax ($K_i \leq 0.5$ nM for BCL-XL and $K_i \leq 1$ nM for BCL-2). On the other hand, cell lines with low or absent BCL-2 protein expression (SUDHL-2, SUDHL-5, and SUDHL-10) had a significantly higher IC50 for

venetoclax (median IC₅₀ 11 μ M) compared to cell lines with high BCL-2 protein expression (OCI-LY3, U-2932, SUDHL-4, SUDHL-6, and SC-1; median IC₅₀ for venetoclax of 0.17 μ M; $p=0.0004$) (Figures 1D and Supplemental figure S3A). In addition, functional BH3 profiling for NOXA ($R=-0.903$; $p=0.005$) and MS1 ($R=-0.756$; $p=0.041$) showed a strong correlation with sensitivity to S63845 (Figure 3C–D), whereas no correlation could be established between the S63845 sensitivity and MCL-1 protein expression (Supplemental figure 3C).

Figure 3. Correlation between BH3 response to BAD, NOXA, and MS1 peptides and sensitivity to anti-apoptotic inhibitors venetoclax (BCL-2i), navitoclax (BCL-2/XL/Wi), S63845 (MCL-1i), and CHOP chemotherapy in DLBCL cell lines. (A) Correlation between venetoclax IC₅₀ versus BAD peptide response (%MOMP). (B) Correlation between navitoclax IC₅₀ versus BAD peptide response (%MOMP). (C) Correlation between CHOP IC₅₀ versus BAD peptide response (%MOMP). (D) Correlation between S63845 IC₅₀ versus NOXA peptide response (%MOMP). (E) Correlation between S63845 IC₅₀ versus MS1 peptide response (%MOMP). (F) Correlation between CHOP IC₅₀ versus NOXA peptide response (%MOMP). Data were plotted as the mean of $n = 3$. Correlation was analyzed using Spearman's correlation. Cell lines without a 'double hit' (MYC/BCL2) status are represented by open symbols (○) and cell lines with a 'double hit' status by closed symbols (●). Cell lines with the highest BCL-2 protein expression (U-2932 and SC-1) are represented by an asterisk (*).

These results demonstrate that, for venetoclax, both BCL-2 protein expression and functional BH3 profiling can predict sensitivity, whereas only functional BH3 profiling can predict sensitivity to S63845. Following these results, we correlated anti-apoptotic protein expression and the functional BH3 profiles to CHOP chemotherapy sensitivity and found that absence of BCL-2 protein expression was associated with low CHOP IC₅₀ (median IC₅₀ 0.44 µg/mL) (Figures 1D and Supplemental figure 3D), while high BCL-2 protein expression was associated with high CHOP IC₅₀ values (median 5 µg/mL). These results are similar to findings in DLBCL patients, in which BCL-2 overexpression (with or without MYC rearrangement) are associated with poor survival in patients treated with R-CHOP (4). This correlation trend was also observed for the functional BCL-2 BH3 profile and CHOP response, in which a high BAD response showed a trend towards high CHOP IC₅₀ values ($R=0.595$; $p=0.1323$) (Figure 3E). No correlation was observed with CHOP sensitivity and MCL-1 protein expression (Supplemental figure 3E) or the functional NOXA BH3 profile (Figure 3F). Taken together, these results show that in untreated/treatment naïve cells, both high BCL-2 expression and functional BH3 profiling for BAD can predict sensitivity to venetoclax and CHOP. However, in the situation that cells show no expression of BCL-2, functional BH3 profiling should be applied to determine sensitivity to specific anti-apoptotic inhibitors.

CHOP Chemotherapy Alters Dependency on Anti-Apoptotic Proteins

Based on previous experiments, we have established that DLBCL cells can express multiple anti-apoptotic proteins simultaneously, but usually show functional dependency on either BCL-2 or MCL-1. However, it remains unclear if standard CHOP treatment has the ability to alter or shift this dependency on anti-apoptotic proteins. To test this hypothesis, we employed dynamic BH3 profiling, which compares the BH3 profiles of treated and untreated cells and plots the differences in response to each BH3 peptide, revealing the altered anti-apoptotic dependencies after specific treatments. Dynamic BH3 profiling of CHOP-treated DLBCL cell lines revealed an enhanced mitochondrial response to the BIM peptide (OCI-LY3, SUDHL-5, SUDHL-6, SUDHL-10, and SC-1), indicating that cells had become more primed for apoptosis (Figure S4).

In addition, five of the eight cell lines (OCI-LY3, U-2932, SUDHL-2, SUDHL-4, SUDHL-6, and SC-1) showed a mitochondrial response to the BAD peptide (Figure 4A), which could indicate that cells had become more dependent on BCL-2 after CHOP treatment. Apart from responses to BAD, three cell lines showed a strong response to the HRK peptide (SUDHL-2, OCI-LY3, and SUDHL-4) (Figure 4B), which could indicate enhanced dependency on BCL-XL, and two cell lines (SUDHL-5 and SUDHL-10) showed a response to the NOXA peptide (Figure 4C), which could indicate enhanced dependency on MCL-1 after CHOP treatment. Combined, these data suggest that CHOP treatment altered the BCL-2 dependency of cell lines OCI-LY3 and SUDHL-4 towards BCL-XL dependency, and made the anti-apoptotic independent cell line SUDHL-2 dependent on BCL-XL (Figure 4D). In the other cell lines, the cell specific anti-apoptotic dependency remained the same, however, it was further

enhanced. None of these CHOP-induced changes were related to a specific cell of origin (COO) or BCL2/MYC status, suggesting that changes are COO and BCL2/MYC independent. Taken together, these data demonstrate that CHOP chemotherapy not only enhances the anti-apoptotic dependency, it can also alter the anti-apoptotic dependency, demonstrating that upfront expression levels of anti-apoptotic proteins are insufficient to predict successful combination with anti-apoptotic inhibitors.

Figure 4. Dynamic BH3 profiles of DLBCL cell lines treated with CHOP chemotherapy. (A) Dynamic BH3 profiles for the DLBCL cell lines OCI-LY3 (1 μ g/mL), U-2932 (10 μ g/mL), SUDHL-2 (1 μ g/mL), SUDHL-4 (10 μ g/mL), SUDHL-5 (0.1 μ g/mL), SUDHL-6 (1 μ g/mL), SUDHL-10 (10 μ g/mL), and SC-1 (10 μ g/mL) after treatment with CHOP for 18 h for BAD, HRK (B) and NOXA (C). Delta mitochondrial outer membrane permeabilization (Δ MOMP%) was calculated by subtracting the percentage treated MOMP from percentage untreated MOMP. Data were plotted as the mean $\pm$ S.D. (n=3). Statistical analysis was performed with a one-sample T-test (* $p \leq 0.05$) (** $p \leq 0.01$). (D) Schematic overview of the anti-apoptotic dependency in DLBCL cell lines before and after CHOP treatment. White = no specific dependency (N.A.), blue = BCL-2 dependency, red = BCL-XL dependency, and green = MCL-1 dependency.

CHOP Chemotherapy Enhances Sensitivity to Anti-Apoptotic Inhibitors

To validate that CHOP treatment enhances or alters the cell specific dependency on anti-apoptotic proteins, we tested the combination of CHOP chemotherapy with venetoclax (BCL-2i), navitoclax (BCL-2/BCL-XL/BCL-Wi), or S63845 (MCL-1i) in the cell lines SC-1 (enhanced BCL-2 dependency), SUDHL-4 (altered BCL-2 $\rightarrow$ BCL-XL dependency), and SUDHL-10 (enhanced MCL-1 dependency).

Treatment with CHOP induced a dose-dependent enhanced sensitivity to venetoclax in cell lines SC-1 and SUDHL-4 (Supplemental figure 5), resulting in a decrease in IC50

values for venetoclax (Figure 5A) and overall strong synergism for the combination of CHOP with venetoclax (Figure 5B). A similar effect was observed when CHOP was combined with navitoclax, which also induced a decrease in IC₅₀ values (Figure 5C) and overall strong synergism (Figure 5D) in cell lines SC-1 and SUDHL-4. These changes were not observed in cell line SUDHL-10, as predicted by dynamic BH3 profiling. Instead, SUDHL-10 showed a strong decrease in IC₅₀ values for S63845 (Figure 5E) and overall strong synergism for the combination of CHOP with S63845 (Figure 5F). Together, these data demonstrate that CHOP chemotherapy can significantly enhance the effectivity of anti-apoptotic inhibitors in DLBCL.

Figure 5. Effect of CHOP and BH3 mimetic combination treatments on viability in DLBCL cell lines. (A) IC₅₀ values for venetoclax and CHOP combination therapy calculated from metabolic activity data in the cell lines SC-1, SUDHL-4, and SUDHL-10. Cells were treated for 72 h and data were normalized to control. Data were plotted as the mean ± S.D. (n=3). (B) Synergism was determined by calculating the combination index (CI) from metabolic activity data for the venetoclax and CHOP combination therapy. Synergism was calculated with the Chou–Talalay method using CompuSyn. Synergistic combinations are depicted in green (CI<1.0), additive combinations are depicted in white (CI=1.0), and antagonistic combinations are depicted in red (CI>1.0). (C) IC₅₀ values for navitoclax and CHOP combination therapy calculated from metabolic activity data in SC-1, SUDHL-4, and SUDHL-10. Cells were treated for 72 h and data were normalized to control. Data were plotted as the mean ± S.D. (n=3). (D) Synergism calculations from metabolic activity data for the navitoclax and CHOP combination therapy (n=3). (E) IC₅₀ values for S63845 and CHOP combination therapy calculated from metabolic activity data in cell lines SC-1, SUDHL-4, and SUDHL-10. Cells were treated for 72 h and data were normalized to control. Data were plotted as the mean ± S.D. (n=3). (F) Synergism calculations from metabolic activity data for the S63845 and CHOP combination therapy (n=3).

In addition to testing the synergy for CHOP together with anti-apoptotic inhibitors, we also investigated whether CHOP treatment would alter the expression levels of anti-apoptotic proteins BCL-2, MCL-1, or BCL-XL (Figure 6).

Despite the increased sensitivity to venetoclax and the changes observed in the dynamic BH3 profiles for the cell lines SC-1 and SUDHL-4, no changes were observed in BCL-2 protein levels after CHOP treatment. Similarly, no changes were observed in MCL-1

protein expression after CHOP treatment in SUDHL-10, despite the strong dynamic BH3 response and enhanced sensitivity for MCL-1 inhibitor S63845. However, protein expression of BCL-XL was increased after CHOP treatment in both SC-1 and SUDHL-4, but not in SUDHL-10. These findings are in line with the observed HRK response in the BH3 profile found in SUDHL-4, although an increased HRK response was not observed in SC-1. These results suggest that while CHOP treatment can change expression levels of anti-apoptotic proteins, these changes may not accurately reflect the dependency of cells on these anti-apoptotic proteins.

Figure 6. Western blot of anti-apoptotic proteins after CHOP treatment in DLBCL cell lines. Western blot of anti-apoptotic proteins BCL-2, MCL-1, BCL-XL, and loading control GAPDH in the DLBCL cell lines SC-1, SUDHL-10 and SUDHL-4 treated with 10 µg/mL CHOP for 24 h.

In conclusion, we were able to validate that CHOP treatment enhances the dependency on anti-apoptotic proteins, without necessarily altering the expression levels of the anti-apoptotic proteins. Importantly, high levels of synergism could be achieved for CHOP with anti-apoptotic inhibitors. Therefore, performing functional assays such as dynamic BH3 profiling is essential to establish how anti-apoptotic dependency may have changed as a result of chemotherapeutic pressure.

Vincristine and Doxorubicin Change Dependency on Anti-Apoptotic Proteins

In previous experiments we demonstrated that CHOP chemotherapy can greatly alter or enhance the dependency on anti-apoptotic proteins, resulting in synergism with anti-apoptotic inhibitors. However, since CHOP chemotherapy is composed of cyclophosphamide (C), doxorubicin (H), vincristine (O) or prednisolone (P), it remains unclear which of these compounds is responsible for the induced effects. We therefore tested each individual compound at the representative dosage of 10 µg/mL CHOP in a clinical ratio of 83/5.5/0.16/11.1, respectively [12] in the representative cell line SC-1 (Figure 7).

Dynamic BH3 profiling of cells treated with vincristine or doxorubicin showed a similar response to the BIM (Figure 7A) and BAD (Figure 7B) peptide when compared to the full CHOP regimen, albeit somewhat lower. The combined effects for vincristine and doxorubicin on BIM (BIM $\Delta 38\% + \Delta 4\% = \Delta 52\%$) were similar compared to the effects induced by CHOP (BIM $\Delta 54\%$), which was also observed for BAD (BAD $\Delta 49\% + \Delta 23\% = \Delta 72\%$ vs. $\Delta 63\%$ by CHOP). No changes were induced by cyclophosphamide or prednisolone. So, although the greatest effect on anti-apoptotic dependency is achieved with complete CHOP, the bulk of the effect can likely be attributed to the actions of vincristine and doxorubicin, indicating that

vincristine and doxorubicin are suitable chemotherapeutics in combination with BH3 mimetic drugs.

Figure 7. Dynamic BH3 profiles of SC-1 treated with individual CHOP compounds. Dynamic BH3 profile of (A) BIM and (B) BAD in the SC-1 cell line treated for 18 h with 10 μ g/mL CHOP, 31.7 μ M cyclophosphamide, 18 nM vincristine, 1 μ M doxorubicin or 3.18 μ M prednisolone. Delta mitochondrial outer membrane permeabilization (Δ MOMP%) was calculated by subtracting the percentage treated MOMP from the percentage untreated MOMP. Data were plotted as the mean $\pm$ S.D. (n=3). Statistical analysis was performed using a one-sample T-test (* $p \leq 0.05$) (** $p \leq 0.01$).

Discussion

While the relationship between BCL-2 expression and CHOP therapy resistance is well established [15,16], it has remained unclear how CHOP treatment affects anti-apoptotic proteins in DLBCL. Using functional BH3 profiling, we showed for the first time that CHOP treatment enhances dependency on anti-apoptotic proteins, making cells more sensitive to BH3 mimetic drugs, most likely through vincristine and/or doxorubicin. We also confirmed that CHOP treatment increases the sensitivity of DLBCL cells to BH3 mimetics, highlighting the potential for clinical applications. Furthermore, our findings highlighted the heterogeneity of anti-apoptotic dependency in DLBCL, in that CHOP treatment resulted in enhanced dependency, not only on BCL-2, but also on MCL-1 and BCL-XL. Since we found similar heterogeneity of anti-apoptotic expression in DLBCL patients, it seems plausible that in patients a similar enhanced or altered pattern on anti-apoptotic proteins might occur. A recent study by Smith et al. confirms this heterogeneity in DLBCL, as they demonstrated in a similar fashion how DLBCL cell lines show specific dependency on either BCL-2, BCL-XL, or MCL-1 for survival [17]. None of the observed changes were related to a specific COO in our DLBCL cell line panel, indicating that initial inhibitor sensitivity or CHOP-induced anti-apoptotic changes might not be related to the differentiation stadium at which DLBCL might occur. In addition, we observed anti-apoptotic changes after CHOP treatment in both 'double-hit' (double MYC/BCL-2 translocation) and non-hit cell lines. It has been recently suggested that BCL-2, but not MCL-1, inhibition is effective in double-hit DLBCL [18]. However, as we have shown that double-hit status does not always match protein expression (e.g., SUDHL-10

lacks BCL-2 expression), this could result in ineffective treatment. In fact, we saw an effective response to MCL-1 inhibitor treatment in the BCL-2/MYC double-hit SUDHL-10 cell line, both prior to CHOP treatment and after CHOP treatment, whereas no response was observed to BCL-2 inhibition. Although our data are broadly in line with recently published clinical trial data for venetoclax in NHL [8], we found a potential impact for MCL-1 dependency after CHOP treatment, which might indicate that a significant number of patients would derive greater benefit from a MCL-1 inhibitor than from venetoclax treatment.

Robust immunohistological chemical staining for BCL-2 expression and the prognosis of R-CHOP treated patients are both well established. However, as this is not the case for MCL-1 and other BCL-2 family members, it is still impossible to predict the prognosis of R-CHOP-treated patients based on the expression of anti-apoptotic proteins. Alternatively, dynamic BH3 profiling would be a very useful tool to predict an anti-apoptotic response to CHOP chemotherapy when applied to treatment-naïve fresh DLBCL patient samples. However, in routine clinical practice, dynamic BH3 profiling of DLBCL samples is challenging, as tissue samples are scarce and dividing cells are required in order to observe possible effects of chemotherapy (e.g., CHOP) on the subsequent BH3 response *in vitro*. *Ex vivo* culture of DLBCL is not possible without stimulation, and stimulation of cells (e.g., CD40 ligand) might skew and alter the BH3 response to chemotherapy. Further research and optimization of BH3 profiling is therefore warranted. In addition, *in vivo* experiments with either a patient-derived xenograft (PDX) model or a spontaneous DLBCL mouse model should preferably be performed to predict and validate the clinical applications of combining CHOP chemotherapy with a broad range of BH3 mimetic drugs.

Besides identifying MCL-1 and BCL-XL as novel contributors to CHOP-induced resistance, our data also show the therapeutic potential of vincristine and doxorubicin in combination with BH3 mimetics. In the current setting, the 10 µg/mL CHOP treatment contained single compound concentrations of 31.7 µM cyclophosphamide, 18 nM vincristine, 1 µM doxorubicin, and 3.18 µM prednisolone, which are all within the range of clinical maximal plasma levels in patients (128 µM cyclophosphamide, 7 nM vincristine, 6.73 µM doxorubicin, and 0.145 µM prednisolone) [19]. At the moment, clinical trials are underway regarding liposomal vincristine and venetoclax in relapsed or refractory T-cell or B-cell NHL (NCT03504644/clinicaltrials.gov). Based on our data, we predict that both vincristine and doxorubicin will induce changes in anti-apoptotic dependency, although whether changes will always favor BCL-2 rather than MCL-1 or BCL-XL will remain unclear without functional dBH3 profiling. In addition, additional experiments with higher concentrations of cyclophosphamide might induce similar anti-apoptotic changes, which warrants further investigation for cyclophosphamide with BH3 mimetic drugs.

In conclusion, we demonstrated the potential of BH3 profiling as a predictor of anti-apoptotic dependencies in DLBCL. Moreover, we showed that CHOP treatment induces increased anti-apoptotic dependency, not only on BCL-2, but also on MCL-1 and BCL-XL. As a result, patient-specific BH3 mimetic treatment might well lead to synergistic lethality and reduce unnecessary treatment. Although BCL-2 remains one of the primary actors in anti-apoptotic resistance, our results indicate that caution is advisable and that other BH3 mimetics should not be neglected as they might well influence or alter therapy outcomes.

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Supporting information

Supplemental Figure 1. Static BH3 profiles of DLBCL cell lines. Static BH3 profiles of DLBCL cell lines, expressed as the percentage mitochondrial outer membrane permeabilization (MOMP%) upon treatment with BH3 peptides ($n=3$). FCCP and PUMA2A were used as positive and negative controls, respectively.

Supplemental Figure 2. Dose-response curves of DLBCL cell lines treated with BH3 mimetics. (A) Dose-response curve of eight DLBCL cell lines treated with the BCL-2 inhibitor venetoclax for 48 hours ($n=3$). (B) Dose-response curve of eight DLBCL cell lines treated with the MCL-1 inhibitor S63845 for 48 hours ($n=3$). (C) Dose-response curve of eight DLBCL cell lines treated with the BCL-2/BCL-XL/BCL-W inhibitor navitoclax for 48 hours ($n=3$). (D) Dose-response curve of eight DLBCL cell lines treated with CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine and prednisolone) for 72 hours ($n=3$).

Supplemental Figure 3. Relation between BCL-2 and MCL-1 protein expression and sensitivity to BH3 mimetics and CHOP chemotherapy in DLBCL cell lines. (A) Venetoclax IC50 plotted based on the absence or presence of BCL-2 protein expression for DLBCL cell lines. (B) Navitoclax IC50 plotted based on the absence or presence of BCL-2 protein expression for DLBCL cell lines. (C) S63845 IC50 plotted based on the absence or presence of MCL-1 protein expression for DLBCL cell lines. (D) CHOP IC50 plotted based on the absence or presence of BCL-2 protein expression for DLBCL cell lines. (E) CHOP IC50 plotted based on the absence or presence of MCL-1 protein expression for DLBCL cell lines. Statistical analysis was performed with two-tailed paired T-test (* $p \leq 0.05$) (** $p \leq 0.01$). Cell lines without a 'double hit' (MYC/BCL2) status are represented by open symbols (○) and cell lines with a 'double hit' status by closed symbols (●). Cell lines with the highest BCL-2 protein expression are represented by an asterisk (*).

Supplemental Figure 4. Dynamic BH3 profiles of DLBCL cell lines treated with CHOP chemotherapy. (A) Dynamic BH3 profiles for the DLBCL cell lines OCI-LY3 (1 μg/mL), U-2932 (10 μg/mL), SUDHL-2 (1 μg/mL), SUDHL-4 (10 μg/mL), SUDHL-5 (0.1 μg/mL), SUDHL-6 (1 μg/mL), SUDHL-10 (10 μg/mL) and SC-1 (10 μg/mL) after treatment with CHOP for 18 hours for BIM. Delta mitochondrial outer membrane permeabilization (ΔMOMP%) was calculated by subtracting the percentage treated MOMP from percentage untreated MOMP. Data were plotted as the mean ± S.D. (n=3). Statistical analysis was performed with a one-sample T-test (* $p \leq 0.05$) (** $p \leq 0.01$).

Supplemental Figure 5. Combined effect of CHOP and BH3 mimetics. (A) Metabolic activity of the DLBCL cell lines SC-1, SUDHL-4 and SUDHL-10 treated with CHOP and venetoclax (BCL-2i) for 72 hours, measured with resazurin. (B) Metabolic activity of the SC-1, SUDHL-4 and SUDHL-10 cell lines treated with CHOP and navitoclax (BCL-2/XL/Wi) for 72 hours, measured with resazurin. (C) Metabolic activity of SC-1, SUDHL-4 and SUDHL-10 treated with CHOP and S63845 (MCL-1i) for 72 hours, measured with resazurin. Data were normalized to the control and plotted as the mean $\pm$ S.D. ($n=3$).

8

Summary, discussion and future perspectives

Summary

Diffuse large B-cell lymphoma (DLBCL) is an aggressive and heterogeneous disease. Current treatment consisting of cyclophosphamide, doxorubicin, vincristine and prednisolone, together with the monoclonal anti-CD20 antibody rituximab (R-CHOP), cures roughly 60% of patients. Unfortunately, this means that many patients will experience relapse or refractory disease and require additional therapy. In this thesis, we aimed to find novel targets to improve current patient treatment and gain more insight into disease development.

In **Chapter 2**, we performed a guilt-by-association analysis for genes co-expressed with CD20, which is both a driver for DLBCL and a target for DLBCL therapy. Further selection based on the presence of clinical compounds resulted in many potential novel targets for treatment. These targets were involved in various different pathways, including the cell cycle regulator protein and the DNA damage protein PARP1. Immunohistochemistry and western blot revealed high expression of WEE1 and PARP in both DLBCL patients and DLBCL cell lines. Moreover, targeting with the clinically available drugs AZD1775 targeting WEE1 and olaparib targeting PARP1 were effective in DLBCL cell lines, indicating their potential for treatment of DLBCL patients. These targets were chosen from the top-500 drugable target list, leaving many targets unexplored. Based on the good results for WEE1 and PARP1, we further explored suitability of other targets involved in similar or different pathways as targets for therapy. At the moment clinical trials are already underway for targeting of CDK1 with seliciclib (NCT00999401) in solid tumors and targeting of HDAC1 with vorinostat (NCT00667615) or panobinostat (NCT00967044) in non-Hodgkin lymphoma, which were all part of our top-500 list. In addition, further research into combination of multiple (top-500) targets could be explored to increase therapy efficiency. Simultaneous inhibition of WEE1 and PARP1 was beneficial for treatment outcome in ovarian cancer, breast cancer [1], gastric cancer [2] and in acute leukemia [3] patients and enhanced sensitivity to radiation therapy in non-small cell lung cancer (NSCLC) [4] and pancreatic cancer [5].

Based on the promising results for treatment with the WEE1 inhibitor AZD1775 as monotherapy and in combination with rituximab in DLBCL cell lines, we next decided to investigate the potential enhanced efficiency of combining AZD1775 with standard CHOP chemotherapy and radiation therapy (RT) in **Chapter 3**. In line with our expectations, combination therapy for WEE1 inhibition alongside CHOP or RT effectively reduced cell viability. Further experiments showed that combination therapy resulted in activation of the DNA damage response, induced premature mitotic entry and increased DNA damage levels, resulting in a strong synergistic effect. Following-up on these data, we decided to investigate the potential for WEE1 in combination with second line therapeutic compounds cytarabine and cisplatin in **Chapter 4**. Similar to our findings for the combination of CHOP and RT, we found that cytarabine strongly reduced cell viability in DLBCL cell lines when combined with AZD1775. To our surprise, the combination of AZD1775 with cisplatin was only effective in one out of four tested DLBCL cell lines, indicating antagonistic effects for this combination. This antagonistic effect was supported by the observation that WEE1 protein

levels were strongly reduced. The reduced expression could be attributed to a reduction of histone marks for active transcription upon cisplatin treatment. Together, these data suggest that cisplatin prevents transcription of the *WEE1* gene, thereby resulting in reduced *WEE1* protein levels. This makes cisplatin an unsuitable partner for combination with *WEE1* inhibitor AZD1775. These data highlight the importance of thorough studies for testing combination treatments, as DLBCL cells might respond differently to a combination treatment shown to be otherwise successful in gastric cancer [6], breast cancer [7] and medulloblastoma [8].

In **Chapter 5**, we tested a yet unexplored combination therapy for AZD1775 together with CDK1/2 inhibitors RO3306 (CDK1i), roscovitine/seliciclib (CDK1/2i) and palbociclib (CDK4/6i). Since both *WEE1* inhibitors and CDK inhibitors are currently being tested in clinical trials for solid tumors and hematological malignancies as monotherapies, we aimed to investigate the potential effect of a combination therapy. Surprisingly, we found that the combination with CDK1 or CDK1/2 inhibitors resulted in either synergism or antagonism in DLBCL cell lines, indicating distinct differences between individual cell lines. Further investigation revealed that these effects were caused by phosphorylation of different checkpoint kinase 1 (CHK1) isoforms, with synergism in cell lines that show activation of the short CHK1 isoform and antagonism in cell lines with the long/normal CHK1 isoform being activated. In other studies, promising results were obtained for the combination of SNS-032 (CDK2/CDK7/CDK9 inhibitor) combined with cytarabine in AML [9] and AZD5438 (CDK1/2/9i) in combination with radiation therapy in non-small cell lung cancer (NSCLC) [10]. Our results pose a difficult challenge and warning for CDK1/2 inhibitor combination therapies: although therapy can be extremely effective in certain cell types, additional studies will be needed to predict effectiveness of therapy outcome and prevent potential harmful effects in part of the patients. Ideally, knock-in experiments should be performed to prove that presence of the short CHK1 isoform indeed results in synergism in therapy-resistant cell lines. It then remains to explain why in some cells the short CHK1 isoform is expressed and activated upon combined *WEE1* and CDK1/2 treatment, and whether this is also observed in DLBCL patients.

A more uniform picture was observed for the combination of *WEE1* with the CDK4/6 inhibitor palbociclib, which induced antagonism in all DLBCL cell lines. In this situation, dosage and timing of the combination therapy were shown to be of the utmost importance in establishing the best possible outcome. Dean et al. showed that the combination of doxorubicin and palbociclib gave better results when breast cancer cells were treated sequentially, rather than concurrently, which resulted in antagonism [11]. In contrast, combination of CDK4/6 inhibitor ribociclib with cisplatin in ovarian cancer was synergistic [12], indicating possible differences between cell types and/or specific inhibitors. Since palbociclib was shown to be antagonistic with many different chemotherapeutics [13], we propose to avoid combining palbociclib with DNA damage inducing therapies. In this scenario more beneficial results might be acquired when CDK4/6 inhibitors are combined with PI3 kinase inhibitors, AKT inhibitors or mTOR inhibitors [14].

Besides focusing on (improving) the existing therapy options for DLBCL, we also investigated the potential new combinations with BH3 mimetics drugs for the treatment of DLBCL, by employing BH3 profiling. In **Chapter 6** we used BH3 profiling to determine the

effect of AZD1775 treatment on anti-apoptotic dependency and found that WEE1 inhibition enhanced sensitivity to multiple anti-apoptotic inhibitors. Interestingly, we showed that induction of DNA damage and/or G2/M cell cycle arrest induced similar changes, suggesting potential efficiency of combining BH3 mimetics with other compounds inducing similar effects. A similar approach was used to investigate the effect of CHOP chemotherapy on anti-apoptotic dependency in **Chapter 7**. Even though it is commonly assumed that anti-apoptotic features/characteristics in DLBCL is acquired through the anti-apoptotic protein BCL-2, we found that cells treated with CHOP therapy became more dependent on the anti-apoptotic BCL-2, MCL-1 and BCL-XL proteins. These data indicate that although BCL-2 is important, other anti-apoptotic proteins such as MCL-1 and BCL-XL should not be overlooked when anticipating therapy resistance or planning targeted therapeutic strategies. The results for WEE1 inhibition and CHOP treatment make it likely that many other DNA damage inducing compounds will induce similar changes in the anti-apoptotic dependency as well. Previous studies showed that cisplatin-resistant lung cancer, cervical cancer and mesothelioma cell lines had enhanced levels of MCL-1, and in a limited number of cell lines BCL-2 [15]. In multiple myeloma cells, treatment with doxorubicin, etoposide and hydrogen peroxide caused upregulation of BCL-2 expression [16]. However, most studies performed have focused on the effect of anti-apoptotic proteins on therapy response, and neglected the opposite relation, i.e. how treatment might affect anti-apoptotic dependency. For example, we learned early on that exogenous overexpression of BCL-2 and BCL-XL protects lymphoid cells against vincristine, vinblastine, gamma-irradiation, etoposide and cisplatin treatment [17]. However, it remains unknown how (long-term) treatments with these compounds affect BCL-2 and BCL-XL expression levels or dependencies in specific normal and/or cancer cell types. This demonstrates the need to investigate the (long-term) effect of new and existing treatment on anti-apoptotic dependency in cancer, and how treatment should be adapted based on the status of these anti-apoptotic proteins. A particularly useful tool in this preclinical research and patient tracking would be the application of dynamic BH3 profiling, as we previously showed that protein expression alone is not always representative of the anti-apoptotic dependency. Grundy et al. screened multiple anti-leukemic drugs with dynamic BH3 profiling in a myelo-monocytic leukemia cell line and showed that 9 out of 11 compounds enhanced sensitivity to the BCL-2 inhibitor venetoclax, and 2 out of 11 enhanced sensitivity to the MCL-1 inhibitor A1210477 [18]. Moreover, they showed that BCL-2 inhibitors themselves enhanced sensitivity to MCL-1 inhibition, indicating a shift in anti-apoptotic dependency. This highlights the power of (dynamic) BH3 profiling for multi-drug screenings, combination strategy planning and potentially long-term follow-up of patient to assess therapy relapses.

Discussion

In this thesis our initial aim was to find novel highly expressed and druggable targets to

improve treatment outcome in DLBCL patients. This resulted in the “discovery” of WEE1 and the experimental support showing the efficiency of WEE1 targeting by various *in vitro* experiments either alone or in combination with other commonly used drugs. Although we have shown that WEE1 is an excellent target for the treatment of DLBCL, one question that remains to be unanswered is: what causes the very high expression of WEE1 in DLBCL, especially compared to other cancers such as breast cancer and lung cancer? A unique feature of DLBCL compared to other cancers is the extremely high expression levels of DNA damage response genes (Figure 1). Although genomic instability is a hallmark of all cancers, gene expression data across multiple cancers shows a unique pattern with high expression for various DNA damage response genes in DLBCL. This specific signature is also present in thymomas, a cancer derived from the epithelial cells of the thymus, a primary lymphoid organ. The relative uncommon thymomas are strongly linked to auto-immune diseases such as myasthenia gravis [19,20]. This overlapping signature might indicate a similar immunological component in DLBCL. However, this pattern of high expression of DNA damage response genes is not observed in acute myeloid leukemia (Figure 1; AML). These studies further point towards the unique DNA damage signature of DLBCL.

Figure 1. Gene expression of WEE1 and DNA damage response proteins in multiple cancer types. Plotted is the factor of tumor / normal tissue gene expression for WEE1 and DNA damage response (DDR) proteins RAD51, PARP1, BRCA1, BRCA2, ATR, ATM, CHK1 and CHK2 in several different cancer types. Gene expression of alpha-fetoprotein (AFP), a plasma protein produced by the yolk sac and the liver during fetal development, is plotted as a negative control. Cancers included are adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), cholangio carcinoma (CHOL), colon adenocarcinoma (COAD), diffuse large B-cell lymphoma (DLBCL), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), acute myeloid leukemia (AML), brain lower grade Glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), mesothelioma (MESO), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumors (TGCT), thyroid carcinoma (THCA), Thymoma (THYM), uterine corpus endometrial Carcinoma (UCEC), uterine carcinosarcoma (UCS) and uveal melanoma (UVM). Data was acquired from the online available GEPIA database (gepia.cancer-pku.cn).

Further reflection on the origin of DLBCL suggests a strong link between the germinal center reaction and the DNA damage response, creating a background for DLBCL that stands out from other cancers. DLBCL is a cancer derived as a result of malignant transformation of B-cells, which either occurs during the germinal center reaction (GCB-DLBCL) or directly after the germinal center reaction (non-GCB/ABC-DLBCL). In a normal germinal center reaction, expression of activation-induced cytidine deaminase (AID) is of crucial importance to induce somatic hyper mutation (SMH) and class switch recombination (CSR) in B-cells [21]. During these processes, DNA damage response (DDR) and repair pathways are activated to ensure proper cellular integrity. This explains the high expression of DDR and DDR-related proteins in germinal center B-cells compared to pre- or post-germinal center B-cells (Figure 2).

Despite activation of proper DDR pathways, the SHM and CSR processes create a

Figure 2. Array gene expression data of DDR proteins during normal B-cell development. B-cells from tonsils ($n=3$) were collected and sorted for naïve, germinal center and memory B-cell populations using flow cytometry. Data was normalized to the internal control HRP gene.

landscape of genomic instability, that may lead to malignant transformation. A crucial role of AID in malignant transformation was supported by the reduced number of malignant transformations in AID^{-/-} mouse models [22] and by the increased number of AID-mediated genomic translocations upon activation of AID in mouse models [23]. Various studies showed that AID is specifically important in the transformation of germinal center-derived lymphomas, and not in pre-germinal center malignancies [22,24,25]. Within the DLBCL patient population, AID expression is higher in the ABC-subtype compared to the GCB-subtype [24,26], which arises from the plasma blast stage of B-cell development representing a more differentiated B-cell. It is therefore not surprising that in DLBCL in patients AID expression is strongly

associated with worse prognosis [27]. In addition, research has shown a close relation between DDR proteins and SHM and CSR processes, such as yH2AX [28], 53BP1 [29] and ATM [30,31]. Moreover, it was shown that inhibition of CHK1 [32] and CHK2 [33] affected AID activity, and as a result SHM and CSR. These studies highlighted the unique landscape of the germinal center, where DDR proteins are often employed in non-canonical activities, independent of their roles in DNA repair [34].

Based on the above-discussed data, we hypothesized that the high expression of WEE1 in DLBCL is likely caused by the AID-dependent induction of DNA lesions, activation of the DDR proteins and a block in cell cycle progression to allow repair. Moreover, the high WEE1 expression may not solely be the result of (extreme) cellular proliferation, but likely also the result of high DDR activation. To study the link between WEE1 and proliferation, we analyzed the relation between the proliferation marker Ki67 and WEE1 in DLBCL patients (unpublished data). No correlation was seen in DLBCL, indicating that WEE1 is not controlled by cellular proliferation in DLBCL (Figure 3A-F). Moreover, correlation analysis between AID and WEE1, or AID with other DDR related proteins also showed no direct correlation (data not shown). Based on these data, it seems difficult to directly correlate AID activity and DDR activation in patients, despite the many mouse models linking AID and DLBCL [22,23]. Instead, it seems more likely that AID activity is part of a (potentially lengthy) process in which multiple events of aberrant translocations and faulty DNA damage repair are together contributing to the malignant transformation of B-cells over a period of time, something which is difficult to track in patient samples.

Figure 3. Expression of WEE1 and Ki67 in DLBCL patients. (A) Gene expression data of WEE1 in DLBCL patient subtypes. (B) Gene expression data of Ki67 in DLBCL patient subtypes. (C) Correlation of WEE1 and Ki67 gene expression in DLBCL patients. (D) Immunohistochemistry of WEE1 protein expression in DLBCL subtypes. (E) Immunohistochemistry of Ki67 protein expression in DLBCL subtypes. (F) Correlation of IHC data for WEE1 and Ki67 protein expression in DLBCL patients.

An important notion to take into consideration is the possibility that DDR pathways might be actively suppressed in the germinal center, as suggested by Ranuncolo et al. [35]. They showed that in order to withstand DNA damage caused by AID, both ATR and CHK1 are actively suppressed by the master transcription factor BCL-6, which is expressed in both normal human centroblasts and DLBCL cell lines [36,37]. Phan et al. showed that BCL-6 actively suppresses TP53 [38] and P21 [39] in Burkitt lymphoma cell lines. This of course functions as a double-edged sword: on the one hand it might prevent TP53-dependent apoptosis and allow cellular proliferation, but at the same time it might also prevent crucial processing and repair of DNA breaks. Similar functions for BCL-6 were also observed in multiple myeloma, where induction of BCL-6 repressed formation of γ H2AX, activated ATM but not ATR, and induced expression of AID [40]. Therefore, an intricate system is at play in the germinal center reaction, in which timing of specific pathways and balance between repair and apoptosis determines the outcome of germinal center B-cells. This balance becomes clear by another study of Phan et al., where they showed degradation of the BCL-6 protein upon DNA damage induction with several chemotherapeutic compounds and radiation [41]. Therefore, it remains difficult to establish exactly how expression of WEE1 is regulated in DLBCL, whether this occurs early or in later stages of the disease and whether this is the cause or the consequence of transformation of germinal center B cells.

Future perspectives

Implication of WEE1 inhibitor treatment for DLBCL patients

Based on our research and the many publications on WEE1 inhibitor treatment in combination with other chemotherapeutic drugs and inhibitors [42], it is generally accepted that the treatment of cancer with WEE1 inhibitors has been a largely successful enterprise. In addition, a high number of clinical trials to test WEE1 inhibition combined with multiple drugs in patients are ongoing, such as fludarabine (NCT02791919) or cytarabine (NCT02791919) in AML, cisplatin in head and neck cancer (NCT02196168), paclitaxel and gemcitabine (NCT02194829) in pancreatic cancer and with carboplatin (NCT01164995) in ovarian cancer. Therefore, we predict that many different types of cancers will benefit from WEE1 inhibition therapy, either as monotherapy or combined with other chemotherapeutics. For DLBCL, combination of AZD1775 with the currently applied R-CHOP treatment, which cures roughly 60% of patients, would most likely improve therapy outcome. However, additional *in vivo* experiments with PDX mouse models need to be done to provide the definitive proof that the combination of AZD1775 with R-CHOP will enhance survival in first line treatment. A similar strategy will be needed for AZD1775 combined with cytarabine, although much more promising clinical evidence for this combination is already available for other tumors, additional clinical trials in DLBCL will provide the necessary efficiency to show the efficiency of this combination treatment.

However, despite promising results for cisplatin and AZD1775 treatment in other cancers [6-8,43-45], platinum-based agents might work antagonistic with AZD1775 in DLBCL, because of the cisplatin-induced repression of WEE1. Further research with other platinum-based agents such as the clinically approved carboplatin, oxaliplatin and nedaplatin, or with compounds that are currently being tested in clinical trials, such as triplatin tetranitrate/BBR3464 (NCT00014547), picoplatin (NCT00465725) or satraplatin (NCT00265655), are required to establish whether the antagonistic effects of cisplatin are specific for this drug or will be observed with all platinum-based agents. In addition, further research into the cisplatin-induced gene repression of WEE1 in DLBCL could elucidate whether these effects are dose-dependent, as suggested by our data. Based on these findings a maximum cisplatin dose could be established for the combined treatment at which WEE1 inhibition does not occur, but tumor cells still experiencing the detrimental effects of DNA crosslinking.

Future research for CDK1/2 inhibitors and CHK1 isoforms

In contrast to the success story of WEE1 inhibitors in cancer therapy, much more research is going to be needed to investigate the possible implications of the different CHK1 isoforms in cancer treatment. Our data gave a first indication that activation of these different isoforms by phosphorylation could be the determining factor for therapy outcome in DLBCL. The short isoform, which is usually less abundant lacks the kinase domain. In addition, Pablo et al. demonstrated that this short CHK1 isoform was strongly associated with cancer and disease progression in ovarian and testicular cancer [46]. They suggested that its “role” in cancer is to inhibit the activity of the normal CHK1 isoform during an unperturbed cell cycle in order to promote S-to-G2/M phase transition. In a situation of DNA damage, phosphorylation of CHK1 could disrupt the interaction between the normal and short CHK1 isoforms, leaving the normal CHK1 free to facilitate cell cycle arrest and DNA damage repair. This would create an ideal situation for cancer cells, in which normal cell cycle progression is maximally stimulated, while in case of DNA damage repair can still be facilitated. However, we observed a clear flaw in this theory: cells which actively phosphorylated the short CHK1 instead of the long CHK1 isoform were less efficiently able to cope with DNA damage, resulting in increased levels of cell death. This slightly compromises the theory that under stress the normal CHK1 isoform will always resume its function, as we never observed simultaneous phosphorylation of the two CHK1 isoforms. However, our data do match with their *in vivo* data, in which xenograft mice expressing only the short CHK1 isoform had significantly less tumor outgrowth compared with mice expressing the normal CHK1 isoform or the CHK1 kinase-dead mutant [46]. Therefore, we hypothesize that although activation of the short CHK1 isoform might be beneficial for unperturbed cell cycle progression, activation of the short CHK1 isoform during cellular stress weakens DNA repair and sensitizes cells to DNA damage inducing treatment. To validate our hypothesis, additional experiments with CDK1 Δ cells could eliminate the possibility of off-target effects of the CDK1/2 inhibitors RO3306 and roscovitine. This would establish a direct link between CDK1/2 inhibition and the CHK1 isoforms, which could be further analyzed with knock-in or knock-out experiments of the

short or normal CHK1 isoforms. Cells actively expressing the short CHK1 isoform could be genetically modified to only express the normal CHK1 isoform to clarify whether this could rescue the cells from fatal DNA damage. Similarly, genetic modification of cells expressing the normal CHK1 isoform, to cells expressing the short CHK1 isoform will reveal whether this sensitizes previously resistant cells to the combination of CDK1/2 inhibitors and DNA damage inducing agents. With these data, we could make a firm, previously undiscovered connection between the cell cycle regulators CDK1/2 and the checkpoint kinase CHK1 in DLBCL.

At the moment, it remains unclear why CDK1/2 inhibition results in activation of the different CHK1 isoforms. It is well known that CHK1 is activated upon DNA damage by phosphorylation of Ser317 and Ser345 by the kinase ATR, resulting in downstream activation of WEE1 and inhibition of the CDC25 phosphatases, in order to block CDK activity and prevent cell cycle progression [47]. Our data suggests a possible feedback loop from CDK1 and CDK2 back to the upstream activation of CHK1, thereby affecting the cellular response to cell cycle arrest. Something similar was proposed by Enomoto et al., who showed that CHK1 was phosphorylated at Ser286 and Ser301 by CDK1 during prophase in order to promote CHK1 translocation to the cytoplasm and stimulate mitotic entry [48]. Similarly, Bacevic et al. showed CDK2 stimulated activation of the ATR/CHK1 pathway upon treatment with hydroxyurea and was required for an efficient DNA replication checkpoint response [49]. In our experiments, inhibition of CDK1/2 seemed to affect activation of CHK1, perhaps through absence of an inhibitory signal from CDK1/2 or as a result of an omitted cytoplasmic translocation of CHK1 as suggested by Enomoto et al. [48]. At the same time, it might well be that currently unknown proteins are involved in the activation of the different CHK1 isoforms. Novel interaction partners of CHK1 might be discovered through co-immunoprecipitation, mass spectrometry or protein microarrays. In addition, our data showed that only CDK1/2 inhibition, but not CDK4/6 inhibition, affected phosphorylation of the CHK1 isoforms. This is quite surprising, since one would expect that when the G2/M phase CDK1 and CDK2 are able to influence CHK1 activity, a similar feedback loop would be in place for the G1/S phase CDK4 and CDK6. In general, it seems the mechanism of CDK4/6 at the G1/S phase of the cell cycle are less well understood compared to their counterparts CDK1/2 at the G2/M phase. For example, we know in great detail the regulating factors for CDK1 and CDK2, which involve: 1) association with their specific cyclins, 2) phosphorylation on Thr160 by CDK activating kinase (CAK), 3) inhibitory phosphorylation by WEE1 on Tyr15 and Thr14 by Myt1 and finally 4) dephosphorylation of Thr14/Tyr15 by the CDC25 phosphatases. Like CDK1/2, CDK4/6 are activated by CAK through phosphorylation on Thr172 and Thr177, respectively, and cellular stress induces phosphorylation of Tyr17 in CDK4 [50] and Tyr24 in CDK6. However, CDK4 and CDK6 are not inhibited by WEE1 [51], but by Scr kinases [52] and their activity is regulated by their assembly with their respective cyclins. These data indicate that CDK4/6 act very different from CDK1/2, and might therefore not be associated with the activation of the different CHK1 isoforms.

At the same time, it remains unclear why activation of the ineffective short CHK1 isoform occurs in certain DLBCL cells, which are biologically capable of efficiently activating the normal CHK1 isoform, whilst it does not occur in other DLBCL cells. Deep analysis of the

differences between so-called “synergistic” and “antagonistic” cell lines could potentially elucidate the differences between these cell lines. One striking observation is the difference in expression of the anti-apoptotic protein BCL-2 between these cell lines. Our protein expression data (Chapter 6 and 7) showed that, with the exception of cell line SUDHL-6, synergistic cell lines lack strong expression of BCL-2, whereas all antagonistic cell lines show strong expression of BCL-2. Several studies have demonstrated a close relation between anti-apoptotic proteins and CDK1/2, where CDK1 is able to phosphorylate BCL-2 [53] and BCL-XL [54] and CDK2 can phosphorylate MCL-1 [55]. The lack of BCL-2 might be an important factor in survival, creating a predisposition in which cells are more sensitive to DNA damage-inducing treatment when combined with CDK1/2 inhibitors. In addition, analysis of other hematological and solid cancers would be required in order to establish whether this phenomenon is unique for DLBCL, or applicable to other cancer types as well. Together, these data will give us new insights in the treatment of cancer with CDK inhibitors and might expose novel fundamental processes of the cancer biology.

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Appendices

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Targeting WEE1 in diffuse large B-cell lymphoma: mediator in DNA damage and apoptosis

Diffuus grootcellig B-cell lymfoom (Diffuse large B-cell lymphoma (DLBCL)) is een agressieve vorm van lymfklierkanker die behoort tot de groep van non-Hodgkin lymfomen. De ziekte ontstaat als gevolg van schadelijke mutaties in de cellen van het humorale immuunsysteem, de B-cellen. Tijdens de normale ontwikkeling van de B-cell ondergaat de B-cell meerdere fases van mutaties en proliferatie in de lymfeklier, om uiteindelijk te differentiëren tot een plasma cell of een B-geheugencel. Echter door foute mutaties in onder andere anti-apoptose eiwitten (bijv. BCL-2) of DNA-schade herstel eiwitten (bijv. TP53) ontsproten deze B-cellen tot DLBCL. Door middel van moleculair onderzoek kan DLBCL verder geclassificeerd worden op basis van het stadium van ontwikkeling in 'germinal centre B-cell like' (GCB), wat ontstaat vroeg in de B-cell ontwikkeling, en 'activated B-cell like' (non-GCB), wat ontstaat later in de B-cell ontwikkeling. Ondanks deze verschillen worden patiënten met DLBCL momenteel behandeld met dezelfde combinatie van verschillende chemotherapeutica (cyclofosfamide, doxorubicine en vincristine) samen met het monoklonale antilichaam rituximab. Rituximab is ontworpen om een interactie aan te gaan met het CD20 molecuul dat tot expressie komt op alle B-cellen, maar extreem hoog tot expressie komt op DLBCL B-cellen. Ondanks dat de huidige therapie genezing geeft voor ongeveer 60% van de patiënten, zijn er veel patiënten die niet reageren op de behandeling of patiënten waarbij de ziekte na verloop van tijd terugkomt.

Om de behandeling voor DLBCL-patiënten te verbeteren, hebben we in **Hoofdstuk 2** een analyse uitgevoerd om nieuwe eiwitten te vinden waarvoor een gerichte therapie kan worden toegepast. In deze analyse hebben we gekeken welke eiwitten samen met het CD20 molecuul hoog tot expressie kwamen in DLBCL-patiënten, o.a. omdat CD20 belangrijk is voor de behandeling met rituximab. In deze analyse vonden we hoge co-expressie van meerdere DNA-schade herstel eiwitten met CD20 in de DLBCL-patiënten, waaronder de eiwit kinase WEE1. De kinase WEE1 is belangrijk voor de regulatie van de celcyclus, en bepaalt wanneer de cell cyclus kan worden vervolgd of moet worden gestopt wanneer er sprake is van ernstige DNA-schade. Behandeling van DLBCL-cellijnen met de specifieke WEE1 remmer AZD1775 zorgde voor een sterke inductie van celdood, en werkte ook goed in combinatie met rituximab. Op basis van deze goede resultaten hebben we vervolgens in **Hoofdstuk 3** verder onderzocht of de specifieke WEE1 remmer goed zou werken in combinatie met bestraling en de chemotherapeutica cyclofosfamide, doxorubicine en vincristine (CHOP) die gebruikt worden in de huidige DLBCL-behandeling. Uit deze experimenten bleek dat de combinatie van AZD1775 met bestraling of CHOP zorgde voor veel extra DNA-schade en verstoring van de cell cyclus, waardoor er sterke synergie optreedt voor de combinatie behandelingen. Vervolgens hebben we in **Hoofdstuk 4** gekeken of de WEE1 remmer ook synergie zou vertonen met cytarabine en cisplatine, twee chemotherapeutica die worden gebruikt bij de tweedelijns behandeling van DLBCL patienten. Uit deze experimenten bleek dat AZD1775 sterke synergie vertoonde samen met cytarabine, maar niet met cisplatine, hoewel dit laatste

wel in de literatuur beschreven was. Eiwit analyse toonde aan dat behandeling van DLBCL-cellijnen met cisplatine zorgde voor een verminderde expressie van het WEE1 eiwit, waardoor de specifieke WEE1 remmer minder effectief was, een fenomeen wat nog niet eerder in de literatuur beschreven was. Op basis van deze gegevens verwachten we dat WEE1 remming goede resultaten zal opleveren in de kliniek als DLBCL-patiënten worden behandeld met AZD1775 in combinatie bestraling, CHOP of cytarabine.

Naast het versterken van de huidige behandelingen, hebben we in **Hoofdstuk 5** onderzocht of de therapie voor DLBCL verbeterd zou kunnen worden door het simultaan remmen van de CDK1 kinase en de WEE1 kinase, eiwitten die beiden hoog tot expressie komen met CD20 (hoofdstuk 2). Voor deze strategie hebben we gekeken naar de remming van WEE1 in combinatie met de CDK1 kinase. Zowel WEE1 als CDK1 kwamen sterk tot expressie in de DLBCL-patiënten genexpressie data, en zijn beiden betrokken bij de regulatie van de celcyclus, waarbij WEE1 de activiteit bepaald van CDK1. Deze combinatie therapie bleek goed te werken in 50% van de DLBCL-cellijnen, waarbij we synergie en extra DNA-schade konden aantonen. Daarnaast was er ook een groep die niet goed reageerde op de behandeling en waarbij we sterke antagonisme vaststelden in de combinatie. Deze data benadrukken o.a. de grote onderlinge verschillen tussen DLBCL-cellijnen, en wellicht ook DLBCL-patiënten. Om te achterhalen waarom DLBCL-cellijnen zo verschillend reageerden op de behandeling hebben we vervolgens gekeken naar de expressie van verschillende DNA-schade eiwitten. Hieruit bleek dat dit verschil wellicht verklaard zou kunnen worden door verschil in expressie van een korte dan wel lange isovorm van het CHK1 eiwit. De korte vorm kwam tot expressie in synergistische cellijnen en de lange vorm in antagonistische cellijnen. Verder onderzoek zal moeten aantonen of deze isovormen inderdaad verklaren waarom de combinatie behandeling van WEE1 en CDK1 remmers kan leiden tot zowel synergisme als antagonisme in DLBCL.

In het tweede deel van dit promotieonderzoek hebben we onderzocht hoe DLBCL-cellen het proces van geprogrammeerde celdood, apoptose, proberen te voorkomen met behulp van de anti-apoptose eiwitten BCL-2, MCL-1 en BCL-XL. Het is algemeen bekend dat mutaties en/of over-expressie van BCL-2 vaak voorkomt in DLBCL-patiënten. Er lopen daarom meerdere klinische trials met de specifieke BCL-2 remmer venetoclax om te onderzoeken of dit middel werkt als mogelijk nieuwe behandeling voor DLBCL-patiënten. Omdat we in eerdere hoofdstukken hebben aangetoond dat WEE1 remming een goede behandeling strategie kan zijn voor DLBCL-patiënten, wilden we daarom onderzoeken of WEE1 remming ook goed zou combineren met anti-apoptotische remmers zoals venetoclax. In **Hoofdstuk 6** hebben we daarom onderzocht wat het effect van behandeling met de specifieke WEE1 remmer zou zijn op de anti-apoptotische eiwitten BCL-2, MCL-1 en BCL-XL. Uit deze experimenten bleek dat remming van de WEE1 kinase zorgde dat de cellen meer afhankelijk werden van de anti-apoptose eiwitten, terwijl de eiwit expressie nauwelijks veranderd. Deze afhankelijkheid was verschillend per cellijn, wat aan geeft hoe verschillend en heterogeen DLBCL-cellen zijn. Als gevolg van deze verhoogde afhankelijkheid waren de cellen hierdoor gevoeliger voor specifieke remmers van BCL-2, MCL-1 en BCL-XL, wat mogelijkheden biedt voor therapie. Daarnaast konden we aantonen dat onafhankelijk zowel DNA-schade als verstoring van de celcyclus, beiden effecten van de WEE1 remmer, zorgden

voor gelijkwaardige veranderingen in de anti-apoptose afhankelijkheid. Als laatste hebben we in **Hoofdstuk 7** gekeken naar de effecten van de chemotherapeutica cyclofosfamide, doxorubicine en vincristine op de anti-apoptose afhankelijkheid in DLBCL. Omdat BCL-2 vaak verhoogd to expressie komt in DLBCL, ligt de focus voor behandeling voornamelijk op de BCL-2 remmer venetoclax, en word er niet of nauwelijks gekeken naar de andere anti-apoptose eiwitten. Daarnaast er is weinig bekend wat mogelijk het effect is van (langdurige) CHOP-behandeling op deze anti-apoptose eiwitten. Met behulp van DLBCL-patiënten materiaal konden we aantonen dat naast BCL-2, ook MCL-1 en BCL-XL vaak verhoogd tot expressie komen in DLBCL-patiënten. Ook zorgde behandeling met cyclofosfamide, doxorubicine en vincristine in DLBCL-cellijnen ervoor dat deze afhankelijkheid werd versterkt of werd veranderd. Hiermee konden we aantonen dat hoewel BCL-2 belangrijk is in DLBCL, andere anti-apoptose eiwitten ook bijdragen aan het voorkomen van apoptose in DLBCL en daarom ook gebruikt kunnen worden voor gerichte therapie in DLBCL-patiënten.

Met dit promotieonderzoek hebben we een eerste stap gedaan om nieuwe mogelijkheden te vinden om de behandeling van DLBCL-patiënten te verbeteren, en daarmee de ziekte beter te leren behandelen. Met behulp van deze resultaten hebben we aangetoond dat remming van WEE1 in combinatie met de huidige therapie, en mogelijk toekomstige therapieën, leidt tot gunstige resultaten en waarschijnlijk een goede strategie zou kunnen zijn voor de behandeling van DLBCL-patiënten. Ondanks dat we goede resultaten hebben behaald met de remming van WEE1, zijn er nog steeds veel andere eiwitten die nog verder onderzocht zouden kunnen worden. Hoewel deze eiwitten wellicht geschikt zijn voor behandeling met specifieke remmers als monotherapie, grondig onderzoek zal nodig zijn om te bepalen of dit zal leiden tot gunstige resultaten in combinatie met andere behandelingen.

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About the author

Mathilde Rikje Willemijn de Jong was born on the 23rd of August 1991 in Utrecht, the Netherlands. She graduated from College de Heemlanden in Houten in 2009. In the same year, she started her University education at Utrecht University, studying Biomedical Sciences. During this period, she studied different fields, including oncology and immunology and took an internship at the lab of Dr. M. Tarsounas at Oxford University. After graduation in 2014, she started her PhD project under the supervision of dr. T. van Meerten and dr. L. Visser in a collaboration between the department of Hematology and Pathology (University Medical Center Groningen). Currently, Mathilde is finishing her research projects and exploring her future career path.

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