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Enhancing Efficacy of Cancer Therapeutics by Targeting Wee1 Kinase

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Scientific Background

Cell cycle events in the human body occur as an ordered series of dependent pathways, whereby each event initiates upon successful completion of the previous ones[1]. Out of the two major phases (interphase and mitotic phase) of cell cycle, interphase consists of the events related to cell growth and DNA replication and mitotic phase separates the replicated DNA and cytoplasmic contents, which leads to the production of two new daughter cells. In order for a cell to move from interphase to the mitotic phase, distinct phases of cell cycle events occur consecutively: G1 phase (first gap) followed by the S Phase (Synthesis of DNA), followed by G2 phase (second gap) and finally M phase (mitotic phase)[1], [2]. When any intra-or extra cellular stress encounters in the cells, the cells are capable of delaying the cell division with the help of checkpoints that maintain the transition of phases of the cycle[1]. Checkpoint mechanism includes various sensors, transducers and mediators that identify the DNA damage and activate series of downstream molecules which causes cell cycle arrest[1]. Thus checkpoints play a mandatory role in maintaining the correct order of cell cycle events and allow cells to respond to DNA damage system.

DNA damage response (DDR) is a complex network of intracellular signaling pathways that senses any damage in the DNA and is involved in cell-cycle checkpoint and apoptosis[3], [4]. This complex machinery consists of different groups of enzymes whose collaborative work helps maintain the genomic integrity by initiating cell cycle arrest and repairing DNA[3]. In response to DNA damage, cell cycle checkpoints get activated in G1, S and G2/M transition phase[5], [6]. Any change in DNA sequence of a gene which codes for one or more regulatory molecules, initiates the loss of control and faulty instructions lead to production of a non-functional protein. Disruption in the monitoring system allows mistakes to be passed on to the daughter cells and with each successive cell division, daughter cells accumulate more damage[2]. When the checkpoint for maintaining cell cycle arrest eventually becomes nonfunctional, unchecked cell division rapidly reproduces abnormal cells that outplace the growth of normal cells in the specific area, resulting in tumor[2].

The DNA repair pathways can work independently or in coordination with other different type of repairing systems to repair the damaged DNA[7]. In response to DNA double strand breaks (DSBs), Ataxia Telangiectasia Mutated (ATM) or ataxia-telangiectasia-related (ATR) protein kinase gets activated; ATM phosphorylates and activates checkpoint kinase 2 (Chk2) that phosphorylates cell division cycle 25 homolog c (Cdc25c) phosphatase and promotes cytoplasmic sequestration of Cdc25c. This induces inhibitory phosphorylation of cyclin dependent kinase 1 (Cdk1)-cyclin B complex, which maintains Cdk1 in an inactive from and prevents entry into mitosis [8]. ATR phosphorylates and activates Chk1, which then phosphorylates Wee1 kinase and Cdc25c, thereby activates Wee1 kinase activity and inactivates Cdc25c. Afterwards, Wee1 phosphorylates and inactivates Cdk1-cyclin B complex, which results in cell-cycle arrest in G2 phase and allows time for repairing of DNA (Figure 1)[8].

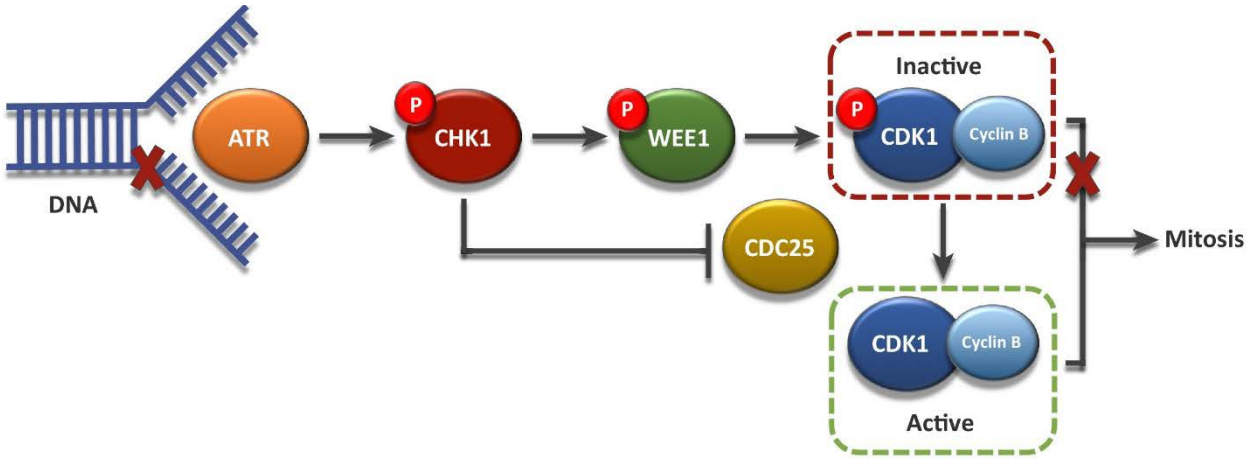

Figure 1. Illustration of the Role of Wee1 in G2/M Checkpoint[8]

ATM induces phosphorylation of p53, reduces its affinity for its negative regulator and leads to p53 stabilization. Stabilized p53 induces p21 and upon protracted checkpoint activation promotes apoptotic cell death[9]. G1 checkpoint is thus critically dependent on p53, but the *TP53* gene is the one most frequently mutated genes in human cancers[7]. The consequence of p53 loss is that the checkpoint in the G1/S transition is compromised and the response to DNA damage now entirely relies on p53-independent mechanisms. In p53-null cancer cells, dependency on the S and G2 checkpoint increases for the repair of the damaged DNA and makes these cells more vulnerable to for anti-cancer agent[1]. The S phase checkpoint deals with slowing the cell cycle rather than the arrest, a cancer cell having DNA damage can bypass the S checkpoint and in such case the G2 checkpoint remains the only keeper of the cancer cell genome and has thus emerged as an attractive therapeutic target for anticancer therapy[1].

The strategy of cancer chemotherapeutic agents or radiotherapy is employing their cytotoxic effects by causing DNA DSBs, which can be recognized and repaired by DNA repairing pathways[7]. The Wee1 tyrosine kinase is one of the main regulator of M-phase Cdks and plays an essential role in maintaining genomic stability by allowing the repair of damaged DNA at G2/M transition[10]. Therefore, targeting the G2/M checkpoint by inhibiting one of its main regulators Wee1, may expand the therapeutic window of DNA damaging treatment in cancer. The potential of Wee1 kinase inhibitor alone or in combination with other drugs can aim for improving the cancer treatment, as well as will help to overcome the treatment resistance to current cancer therapeutics.

Therefore, this article will serve to review the biology of checkpoint kinase Wee1, inhibition of Wee1 as an effective measure of abrogating G2 arrest, its suitability as target in cancer therapeutic, current Wee1 inhibitors in use, and the potential of other Wee1 inhibitor for the improvement of cancer therapy in future.

Wee1 Kinase

Discovery of Wee1 Kinase

The *Wee1* gene product was first identified in fission yeast *Schizosaccharomyces pombe* that is capable of phosphorylating Tyr-15 in Cdk1[11]. The name was given such because of the 'wee' phenotype in fission yeast, which caused delay in mitosis [12]. Afterwards, homologs of Wee1 kinase have been sequentially identified in a wide range of species including human, frog, mouse, *Saccharomyces cerevisiae* and *Drosophila* [11]. In vertebrate system, Thr-14 in Cdk1 also gets phosphorylated, but the kinase responsible for this phosphorylation is membrane-associated kinase, the Myt1 kinase, which was shown to be capable of phosphorylating Thr-14 and to a minimum extent Tyr-15 in Cdk1 as well[11]. Fission yeast Wee1 is a large protein with a molecular mass of ~10 kDa and the kinase catalytic domain is confined to the ~35 kDa of C-terminal domain[13]. The activity of fission yeast Wee1 is downregulated by protein phosphorylation, most likely by the kinases Cdk1, Cdr1/Nim1 and Cdr2[13]. The human *WEE1* gene is located on 11p15.3-p15.1 and contains 646-amino acid. Among the 3 domains, that are *N*-terminal and C-terminal regulatory domain and a central kinase domain; the kinase domain is responsible for the Cdk1 phosphorylation *in vivo* [14].

Regulation of Wee1 Activity

The Cdk complexes are the effectors of cell cycle progression that consist of a catalytic kinase subunit and a regulatory chain[15]. The Cdk/cyclin complexes are activated sequentially during cell cycle which drives the cycle by phosphorylating different target substrates. The activity of the complexes is tightly regulated at multiple levels. Wee1 family kinases negatively regulate the Cdk complex through phosphorylation of Tyr-15 residue on Cdk1 (Cdc2), which inhibits binding of ATP and block the recognition of its target substrates[15]. Wee1 kinase works in antagonism with Cdc25c that helps in removing the inhibitory phosphorylation and promotes entry of cells into mitosis. Both Wee1 and Cdc25c are highly conserved which suggests that their mechanism of controlling the entry of cell into mitosis is similar in all eukaryotic cells[16].

Figure 2 describes the role of Wee1 as a key mitotic inhibitor and its regulation in the intricate network of phosphatase and kinases that regulate mitotic entry[14]. Wee1 and Cdc25 constitute the main switch for mitotic entry, and this switch is regulated by post-translational modification[14]. This mechanism consists of double-activating feedback loops, where activated Cdk1 activates Cdc25 and MastL (activators) and inactivates Wee1 and Myt1 (inactivators) to push the cycle towards mitosis (Figure 2). Three parallel Cdk1 inactivating pathways maintain G2 arrest: Chk1/Wee1/Cdc25/Cdk1, Myt1/Cdk1, and PP2A/Wee1/Cdc25 (Figure 2). Inhibitors of Wee1, Chk1, Hsp90 or PP2A trigger onset of mitosis[14]. Moreover, the positive regulation of mitosis is mediated by PI3K/Akt, Aurka/Plk1, MastL/PP2A, Kruppel-like factor 2 (KLF2), Pin1 etc, and mitosis is negatively regulated by inhibitors of Akt, Cdk1, Plk1, or Aurka (Figure 2)[14].

The Wee1 kinase is controlled by various mechanisms. Phosphorylation of Wee1 by Cdk1 and polo-like kinase 1 (Plk1) produces phosphodegrons for β-TrCP F-box protein-containing SCF E3 ubiquitin ligase and signal ubiquitination of Wee1 by Cdc34 thus induces proteasome-dependent degradation required for initiation of mitosis (Figure 2)[17]. Moreover, Wee1 phosphorylation at S642 by Akt1 creates 14–3-3θ peptide-binding site that decreases its level of activity[14]. KLF2, a Cys^2/His^2 zinc-finger transcriptional factor, negatively regulates Wee1 expression (Figure 2). Binding of KLF2 to Sp1/CPBP motif on Wee1 promoter causes repression of Wee1 basal promoter activity and decreases Wee1 mRNA and protein levels[18]. The circadian rhythm of the clock also controls Wee1 expression[14]. Wee1 can also be downregulated at the transcriptional level by CCAAT/enhancer-binding protein β (C/EBPβ) which acts as mitosis inhibitor protein[19].

Wee1 activity is positively regulated by Chk1 and 14-3-3 proteins (Figure 2)[14]. Binding of 14-3-3 proteins to Cdc25 sequesters Cdc25 in the cytoplasm before mitosis and for this binding, Cdc25 should be phosphorylated by kinases i.e. Chk1[20]. 14-3-3 proteins regulate Cdc25 by promoting the retention of Cdc25 in the cytoplasmic compartment during interphase and this retention by 14-3-3 proteins require intact 14-3-3 binding motif[21]. PP2A and Hsp90 are also the positive regulators of Wee1 (Figure 2). Wee1 must be deregulated rapidly at the onset of mitosis to activate Cdk1. Therefore, Phosphorylation of Wee1 by Cdk1 at S53 and Plk1 at S123 can target Wee1 for F-box proteins SCF–β-TrCP ubiquitin ligase-mediated proteasomal degradation[17].

In malignant melanoma, Wee1 is directly regulated by miR-195. miR-195 mediated downregulation of Wee1 in metastatic lesions can help to overcome cell cycle arrest under stress conditions[22]. Wee1 can interact with the anaphase promoting complex, functioning as a negative regulator. The anaphase-promoting complex/cyclosome (APC/C), a multi-subunit member of the RING finger family of ubiquitin ligases, has a vital role in the regulation of mitotic progression[23]. Wee1 and APC/C constitute a negative feedback loop which regulate each other during cell-cycle progression, i.e., APC/C mediates Wee1 degradation to promote mitotic progression, while Wee1 inhibits APC/C activity to delay this progression to maintain genomic integrity[23].

Checkpoint kinases maintain a positive/negative regulation with other cellular proteins and the interaction changes when any stress alters the equilibria of cell. Phosphorylation is critical for the regulation of cell cycle events. Chk1, 14-3-3, PP2A and Hsp90 proteins act as positive regulators of Wee1 and Akt1, KLF2, C/EBPβ on the contrary act as negative regulators. Wee1 inactivation can occur by phosphorylation and degradation both, i.e. SCF–β-TrCP causes ubiquitination of Wee1 when Wee1 in phosphorylated by Cdk1 and Plk1. Cdks play essential role in cell cycle control and G2/M transition. During mitotic entry, Wee1 activity is decreased by various regulators and Cdk1 activity increases. Cdk1 activity is balanced by inactivating phosphorylation by Wee1 and Myt1 and by activating dephosphorylation by Cdc25.

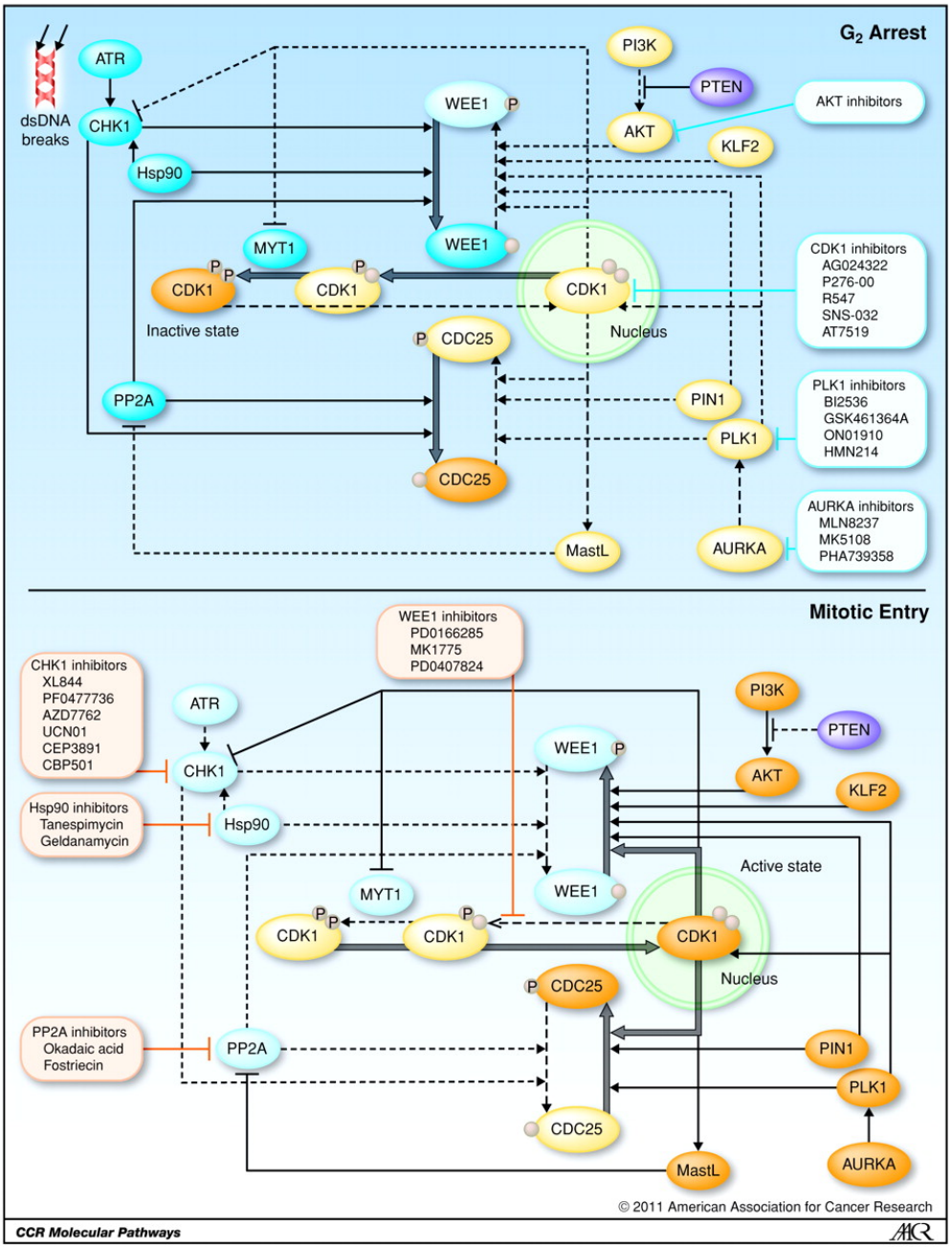

Figure 2. Positive and negative regulation of Wee1 [14]. Mitotic activator are indicated in orange and inactivators are indicated in blue.

Wee1 kinase as Target for Anticancer Therapy

Upregulation of Wee1 in cancer

Wee1 kinase is a vital regulator of G2 checkpoint and gene expression profiling has revealed that it is upregulated in various cancer cells i.e. glioblastoma, colon cancer, seminoma, breast cancer,

osteosarcoma, ovarian cancer and many other cancer types[24]. High expression of Wee1 has been shown to be related with poor-disease free survival[25]. Overexpression of Wee1 is associated with decrease in histone level which leads towards insufficient chromatin packaging; this makes DNA more accessible to damage repair machinery and promote radioresistance[26]. The capability of Wee1 to downregulate level of histone explains the reason of dependency of cancer cells on its epigenetic activity[26]. Along with the acquired radioresistance, decreased nucleosomal packaging causes local alterations in chromatin architecture which can activate transcription of oncogenes, that otherwise remains in check in normal cells. Inhibiting Wee1 alone in actively replicating cancer cells has shown to increase histone level and in combination with DNA damaging agents could interfere with DNA repair machinery[26].

The altered expression of Wee1, its impact on disease progression and clinical outcome varies with the cancer types. Upregulation of Wee1 follows tumor progression and is correlated with thicker tumor, ulceration and decreased relapse free survival in benign nevi and primary – and metastatic melanoma, glioblastoma and breast cancer[27]. Wee1 also has a positive correlation with markers of proliferation like Cyclin A, Ki67 and Cyclin D3[28]. Another mechanism of Wee1 regulation in response to cancer is posttranscriptional gene regulation[29]. HuR, a key RNA binding protein in cancer, binds to target mRNA containing AU- or U-rich (ARE) elements in their 3′ untranslated region and upon specific stress, posttranscriptionally regulates specific mRNAs. HuR stabilizes Wee1 mRNA and Wee1 protein expression is posttranscriptionally regulated by HuR upon DNA damage[29].

Inhibitors of Wee1

There are a number of small molecules that can inhibit Wee1. These compounds are based on pyrimidine and pyrrolo-carbazole derivatives and the working principle of these compounds is to abolish Cdk1 phosphorylation on Tyr-15[14]. Preliminary clinical studies also revealed that Wee1 inhibitors, when used alone, are well tolerated by the body with acceptable adverse effects[14]. The search for G2 checkpoint abrogator led the development of a pyrido [2,3-d] pyimidine compound, PD0166285, that is although potent, but nonselective Wee1 inhibitor as it targets other kinases as well such as Myt1, epidermal growth factor receptor (EGFR), fibroblast growth factor receptor 1 (FGFR1), Chk1 etc, but can inhibit Wee1 at nanomolar concentration[30], [31]. It inhibits Chk1 kinase, but does not inhibit Cdk1/cyclin B. In several cancer cell lines, short exposure (2-4 hr) to PD0166285 at 0.5 μM concentration dramatically inhibited radiation induced Cdk1 phosphorylation[30]. It was observed that PD0166285 is capable of abrogating G2 checkpoint and can sensitize HT29 (human colon carcinoma cell line with p53 mutation) cells to radiation based therapy[30]. Thus, PD0166285 acts as a radiosensitizer promoting cell death[32]. Another pyrrolo-carbazole derivative, PD0407824, is more selective but less potent inhibitor of Wee1[31]. Other kinase targets of this inhibitor are Cdk4, Akt, Chk1 and FGFR, which are inhibited at much higher concentration that normally used for Wee1 inhibition[31].

The pyrazolo-pyrimidinone AZD1775 (also known as MK1775) is a potential ATP-competitive inhibitor of Wee1 kinase activity[33]. It is highly selective and it can effectively suppress the kinase activity of recombinant human WEE1 (IC_{50} = 5 nM) by successfully competing with the ATP-binding site[34]. Several compounds bearing modifications of the aniline substituent has resulted as an equal potent Wee1 inhibitor like AZD1775. The *N*-methylpiperazine of AZD1775 is amenable to modification and the additional flexibility in the alkylamino side chain allows better ATP-binding capacity which can improve the inhibitory capacity[33]. Replacement of the allyl group at the pyrazole N^2 position can prove as a potential alternative strategy, however, further investigation is needed to develop novel compounds which elucidates the scope of replacing allyl group that will include substituents that can be accommodated in the small hydrophobic pocket of ATP-binding site of Wee1[33]. The working principle of AZD1775 of cell growth inhibition is mainly attributed to either DDR or premature mitosis[35]. Some toxicity with Wee1 inhibitor occurred in animals in lymphoid, hematopoietic, and gastrointestinal systems, however, the toxicity level is reversible[36]. AZD1775 is the kinase inhibitor that is currently in clinical development[36]. Another Wee1 inhibitor CJM061, demonstrated reduced cytotoxicity in comparison to AZD1775, giving a notion about the scope of developing pyrazolopyrimidine-based Wee1 inhibitors with better selectivity and less toxicity profiles[8].

Among the Wee kinase family, Wee1B is less important in terms of drug target and Wee1 and PkMyt1 holds importance on the counterpart. Wee1 and PkMyt1 are kinases that play vital roles in DNA-damage recovery[37]. In small cell lung cancer, low expression of PkMyt1 could predispose cancer cell to Wee1 inhibitor AZD1775 sensitivity, as PkMyt1 lowered the threshold of kinase inhibitor[38]. However, cell-based PkMyt1 assays still remains pending which may open up further opportunities to develop new PkMyt1 inhibitors.

One of the major challenge in the development of kinase inhibitor remains in the achievement of kinase selectivity as they are often designed to target ATP-binding site of kinases and may cross-react with other kinases within the kinome and inhibit them[39]. Kinome profiling is therefore performed on lead kinase inhibitors to evaluate their ability to inhibit other kinases. A desirable property of kinase inhibitor is kinase selectivity to enable the highest impact of inhibition and to limit off-target effects or toxicities that is unrelated to target kinase inhibition[8]. Moreover, because of the tumor microenvironment, kinase inhibitors that sensitize cells *in vitro*, face additional challenges in killing cancer cells selectively *in vivo*[40]. The difference in growth kinetics, intratumoral heterogeneity and drug delivery *in vivo* influence the efficacy of the inhibitors. Injury to normal tissue also imposes a great concern[40].

Among all the molecules that serve the function of Wee1 inhibitor, AZD1775 is the most potential in terms of efficacy, as well as selectivity. There is evidence of AZD1775 suppressing the Wee1 kinase effectively, with protein level phospho-Cdk1 decreased. In preclinical and clinical studies, AZD1775 has already showed its efficacy in reducing various cancers as single agent, or in combination therapy. It has been found to be useful in radio and chemo-sensitizing tumors to radiation and chemo therapeutic agents, which are discussed later in the article.

Monotherapy with Wee1 inhibitor *in vitro*

Due to overexpression of Wee1 in malignant cancer, Wee1 inhibition has become an advantageous strategy for survival against cancer. Many *in vitro* studies have demonstrated the cytotoxicity of Wee1 inhibitor in cancer cell lines. Adavosertib (known as AZD1775) has shown promising result as single-agent in clinical trials against multiple solid tumors including glioma, sarcoma, ovarian cancer, head and neck cancer[41], [42]. It also showed significant potentiality in combination with chemotherapy and ionizing radiotherapy. Adavosertib treatment in cervical cancer cells (HeLa) and breast cancer cells works by directing S-phase cells towards mitosis causing premature condensation of chromosomes that are not properly replicated. This leads to double strand breaks at the centromeres, cell-arrest and death in prometaphase[43]. Inhibition of Wee1 in gastric cancer cells with highly expressed Wee1 decreased cell viability and invasion, whereas Wee1 overexpression reversed the effect in gastric cancer cells with low Wee1 expression, suggesting the regulation of cell proliferation, viability and invasion of gastric cancer cells are enhanced by overexpression of Wee1[44]. Inhibition of Wee1 by its inhibitor AZD1775 in gastric cancer cells demonstrated higher killing sensitivity of AZD1775 in p53 wild type gastric cancer cells. In acute lymphoblastic leukemia (ALL), Wee1 is significantly present in adult cell sample.

AZD1775 has been used as a single agent and has been found to effectively reduce cell viability in a panel of eight ALL cell lines[45]. AZD1775 is effective as a single agent therapy in ovarian cancer, independent of p53 status[46]. However, p53 defect may sensitize ovarian cancer cells to AZD1775[46]. In sarcoma cells, AZD1775 treatment leads to unscheduled $Cdk1^{Y15}$ activation and cell death in sarcoma cells[47]. Treatment with AZD1775 also causes increased serine 139 phosphorylation of H2AX, which indicates that Wee1 inhibition causes DNA damage in the cells and consequently mitotic catastrophe by allowing sarcoma cells with DNA damage enter into mitosis[47]. In esophageal squamous cell carcinoma (ESCC), the expression of Wee1 is found much higher in the cell line and in clinical samples as well compared to the corresponding control[35]. AZD1775 suppressed the wound healing capacity of ESCC cell lines and dramatically inhibited their invasion and migration. It significantly downregulated the expression of MMP-2 and MMP-9, which are two critical players of tumor-invasion and metastasis in many malignant cancers including ESCC[35].

In p53 wild-type melanoma cells, mono-therapy with Wee1 inhibitor PD0166285 caused a shift of cells from G2 to G1 arrest and downregulated the proliferation[32]. With PD0166285 treatment, melanoma cells dramatically abrogated G2 checkpoint and were seen to arrest in the G1 phase by completely abolishing Cdk1-Tyr15 phosphorylation[32]. This effect of Wee1 inhibitor for G1 arrest demonstrates the activity of this kinase in an unusual way. In colon cancer cells, combination with radiation therapy with PD0166285 increased the number of mitotic cells, while the effect was minor in monotherapy[30]. These studies clearly indicate the role of Wee1 inhibition in cancer cell death via apoptosis, consequently highlighting the potential of AZD1775 as a single agent therapy.

In vivo and *ex vivo* studies with Wee1 inhibitor

Wee1 inhibition has shown promising results in tumor growth reduction using xenografts animal models in various studies. AZD1775 treatment in patient derived sarcoma tumor xenografts could result in apoptotic cell death, when tumor explants were treated with the inhibitor *ex vivo*[47]. *Ex vivo* treatment of the cells resulted in activation of Cdk1 in the samples along with $Cdk1^{Tyr15}$ inhibition. In esophageal cancer (ESCA), Wee1 inhibition effectively radiosensitized ESCA cells in mice[48]. AZD1775 leads to promotion of cell death through mitosis, despite the DNA damage from the radiation and leads to drastic radiosensitization by this Wee1 inhibitor *in vivo*. AZD1775 greatly attenuated the tumor growth of xenografted ECSS cells in nude mice and could suppress the expression of phospho-CDK1 (Y15)[35]. In an established mouse model of orthotopic human gastric cancer, which mimics the physiology of human gastric cancers, there was a suppression of tumor growth in mice treated with AZD1775 alone[44]. The toxicity profile was also analyzed and there was no sign of weight loss in the mice that received AZD1775. Moreover, enhanced treatment outcome was seen when AZD1775 was administered in mice in combination with anticancer agents 5-Fluorouracil (5-FU) and paclitaxel[44]. In another study with patient-derived pancreatic carcinoma xenograft models, the groups that received single agent treatment of Wee1 inhibitor AZD1775 at a dose lower that the maximum tolerated concentration, showed surprising tumor growth inhibition rate in the xenografts[49].

The *ex vivo* and *in vivo* studies support the idea of inhibiting tumor cell growth and metastasis by AZD1775. The toxicity profile assessed in the studies could reveal the less amount of toxicity associated with this kinase inhibition in comparison with other traditional therapeutic agents used against cancer, which is known to have severe side effects. The promotion of cell death and inhibition of tumor growth warrants further investigations to evaluate the efficacy of Wee1 inhibitor AZD1775 for the treatment of cancers where it is currently being used now, and also for other types of cancer where Wee1 kinase is found to be upregulated and is associated with metastasis.

Setbacks of Wee1 Therapy

Although Wee1 inhibitors have already reached advanced stage clinical trials, determinants of sensitivity of Wee1 inhibitors are less known. One study has proposed a synthetic lethal interaction between loss of histone H3K36me3 and inhibition of Wee1[50]. H3K36me3 is frequently lost in many cancer types. To target this lethal interaction, a subunit of ribonucleotide reductase, RRM2 was selected, which is regulated by two pathways in this context (Figure 3). The first pathway works by H3K36me3 depletion catalyzed by tumor suppressor SETD2 which promotes RRM2 expression. Disrupting the second pathway by Wee1 inhibition leads to Cdk activation that promotes RRM2 degradation, reduces dNTP pools, inhibits DNA replication and causes cell death[50]. H3K36me3 depletion thus serves as a determinant of sensitivity towards Wee1 inhibition and Wee1 inhibitors can selectively kill H3K36me3-deficient cancers.

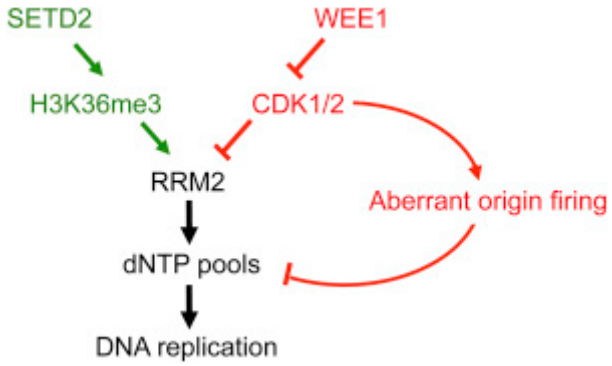

Figure 3. Synthetic lethal interaction between H3K36me3 depletion and Wee1 inhibition[50]

Cyclin E, a key regulator of G1/S transition can activate Cdk2 to progress towards S phase and cyclin E levels are tightly regulated in cell cycle of normal cells[51], [52]. Cyclin E overexpression has been observed in human malignancies and is associated with poor outcomes[53]. An overactive cyclin E/Cdk2 complex causes DNA replication stress in cancer cells and can sensitize these cells to Wee1 kinase inhibition[53]. On the other hand, Cdk2 deletion makes the cancer cells resistant towards Wee1 inhibition. Triple-negative and basal-like breast cancers (TNBCs) with cyclin E overexpression show much more sensitivity towards AZD1775 than the cyclin E low TNBC tumors[53]. Cyclin E high cohort is likely to respond to mono-therapy of AZD1775. Thus Cyclin E can be used as a biomarker to measure the sensitivity of cancer cells towards Wee1 inhibition.

Resistance to Wee1 Inhibition

Despite the very specific mechanism of Wee1 inhibition, some tumors do not respond to AZD1775 (adavosertib) in the clinical trials[41], [54]. In eukaryotes, Wee1 and Myt1 kinase has functional redundant role in the inhibition of Cdk1/cyclin B complex and because of this functional redundancy, compensatory Myt1 activation works as a possible mechanism for resistance towards AZD1775 [43]. In a subset of glioblastoma cells, Myt1 has been shown to play a vital role in survival of cells, which had downregulated Wee1 expression and loss of Myt1 in these cells caused mitotic arrest[55]. Compared to Wee1 knockdown alone, combined knockdown of Wee1 and Myt1 increases the number of HeLa cells entering mitosis with damaged DNA[56]. Upregulation of Myt1 can mediate intrinsic and acquired resistance in breast cancer model to AZD1775 by inhibiting ectopic Cdk1 activity[43].

Mutational status of various S phase genes, i.e. *Cdk2*, can determine the cytotoxicity caused by Wee1 inhibition[56]. Cdk2 is also phosphorylated by Wee1 on Tyr-15 thus preventing unscheduled S-phase entry. As most of the tumors lack functional p53, *TP53* mutant tumors depend on Wee1 inhibition for genomic stability, but studies indicate that only a subset of the p53 mutant patient derived pancreatic cancer xenograft shows sensitivity towards Wee1 inhibition and the rest remained resistant[49]. This indicates that there are other genetic determinants for Wee1 inhibitor sensitivity than the *TP53* mutational status[49]. The involvement of Wee1 in S phase provides an

explanation of Wee1 inhibition resistance in S-phase regulatory gene mutants. Expression levels of S-phase regulators i.e. Cul1, Skp2, and Cdk2 are associated to varying degree with sensitivity towards Wee1 inhibition in *TP53* mutant tumor cells[49]. The G_2-phase abrogation induced by Wee1 inhibition can be rescued by inactivation of these identified S-phase regulatory genes[49].

Small-cell lung cancer (SCLC) is the most aggressive form of lung cancer which develops rapid drug resistance[57]. AZD1775 has been proved as a potent agent in clinical trial in a subset of SCLC patients, but treatment resistance against AZD1775 remains a common phenomenon[58]. AXL, a receptor tyrosine kinase that interacts with growth arrest-specific protein 6, is frequently overexpressed in various types of cancer that have undergone EMT[59]. This receptor tyrosine kinase is a driver of cellular processes that are critical for growth, development, spreading of tumor, and is also responsible maintaining cancer stem cell properties[59]. AXL promotes resistance to Wee1 inhibition via downstream mTOR signaling and resulting activation of Chk1[58]. After the development of acquired AZD1775 resistance, upregulation of AXL and MET, a second tyrosine kinase, occurs. This acquired resistance can be reversed by simultaneously inhibiting Wee1 and AXL/MET or mTOR[58]. Thus, co-targeting AXL or mTOR is a good strategy to overcome Wee1 inhibitor resistance in SCLC models. The combined Wee1/mTOR inhibition is also a promising treatment option for epithelial ovarian cancer patients and overcomes the primary and acquired resistance to Wee1 inhibition alone in this cancer type[60].

Combination of Wee1 Inhibitor with Other Drugs

Novel combination therapies with Wee1 inhibitors have gained interest in recent years as they can overcome treatment resistance to classic radio and chemotherapy in different types of cancer. The *in vitro* and *in vivo* studies suggest some outstanding outcome with Wee1 inhibitor when combined with radioactive agent and chemotherapeutics or with other checkpoint kinase inhibitors, which as single agents, couldn't provide the expected clinical outcome.

Wee1 inhibition with radio- and chemotherapeutic agents

Provided with the potential preclinical results demonstrating enhanced cytotoxicity of Wee1 inhibitor with various DNA damaging agents, Wee1 inhibition has been tested as a radiosensitizer and chemo-toxicity enhancer in human tumor cells derived from various form of cancer. The incorporation of AZD1775 into radiation therapy or being combined with DNA damaging agents has emerged as an attractive anti-cancer treatment strategy which is currently being evaluated in clinical trials of a wide range of cancers (http://www.clinicaltrials.gov). The first line treatment for diffuse large B-cell lymphoma (DLBCL) for several decades has consisted of the chemotherapy combination of agents cyclophosphamide, vincristine, doxorubicin and prednisone (CHOP) together with the anti-CD20 antibody rituximab (R), with or without radiation therapy (RT). Although, 30-40% patients experience disease relapse and 10% of the patients develop primary refractory disease. Wee1 acts as a potent target in DLBCL and showed its efficacy in combination with rituximab[61]. The combined effect of Wee1 inhibitor AZD1775 with CHOP and RT against

DLBCL can activate the DDR pathways, limit the time necessary for repairing of DNA, induce premature mitotic entry which ultimately leads to synergistic lethality of DLBCL cells[61].

Wee1 inhibition in combination with RT has also proven successful in gliomas and osteosarcoma. Synergistic effect of AZD1775 with other chemotherapeutic agents has also been successful in other cancer types, i.e. with doxorubicin in colon cancer and B-cell lymphoma cell lines, with vincristine in B-cell and T-cell leukaemia cell lines as well as patient cells, with cyclophosphamide-like compounds in lymphoblastoid and rhabdomyosarcoma cell lines, with paclitaxel in breast cancer cells and with cytarabine in B-cell lymphoma cell lines and xenograft mouse models[61]. In ovarian cancer cells, Wee1 inhibitor AZD1775 induced minor cell apoptosis whereas when combined with gemcitabine, the cell death increased dramatically[34]. In a glioblastoma cell line, mono treatment with AZD1775 inhibited growth of cancer cells to a lesser extent and combining irradiation could give better result[62]. In p53 deficient lung, colon and pancreatic cancer cell lines with AZD1775 alone or 5-fluorouracil (5-FU) alone could not reduce viability of cells but when the treatment was combined, the effect was strongly enhanced[49]. Diffuse intrinsic pontine glioma (DIPG) cells showed moderate reduction in cell viability in response to AZD1775 monotherapy, combination with irradiation demonstrated increased cell death[63]. Osteosarcoma cells which are normally resistant to radiotherapy, showed increase in number of apoptosis when treated with Wee1 inhibitor PD0166285 and radiation therapy both, but monotherapy with PD0166285 alone did not show any effect[64].

The role of immune system in tumor eradication has gained interest in recent years. Defective apoptosis of tumor cells express high level of brachyury, a T-box transcription factor, which facilitates tumor dissemination and resistance to chemotherapy and radiation by activating epithelial–mesenchymal transition (EMT)[36]. Along with the EMT, the properties of cancer stem cells (CSC) are also responsible for chemo-resistance and recurrence after therapy[65]. Wee1 is capable of maintaining a stem-like state and therapy-resistance in cancer cells. High levels of brachyury reduces the susceptibility of tumor cells to either antigen-specific $CD8^+$ CTLs or innate natural killer (NK) and lymphokine-activated killer (LAK) cells. Pretreatment of tumor cells with Wee1 inhibitor can counter the defective apoptosis of tumor cells, thereby reconstituting the Cdk1 activity to such a level that is sufficient to improve immune mediated attack of brachyury-high tumor cells[66].

Radiation therapy and chemotherapy are conventional anticancer treatment, however, their efficacy is controversial in certain types of cancer because of the disease relapse and the development of resistance in patients towards these therapies. Novel small molecule, like Wee1 inhibitor serves as radio and chemosensitizer. In sync with the functions of these therapies in cancer, Wee1 kinase inhibitor abrogates G2 checkpoint in cancer cells, leading the cells towards premature mitotic entry and sensitize the cells to apoptosis by these anticancer agents. Optimal treatment efficacy is obtained with less time with the combination therapy. Wee1 imbibition in

combination with DNA damaging treatments has demonstrated promising preclinical outcomes which has led it towards combination treatments in advanced phase clinical trials.

Novel combination strategies with Wee1 and other kinase inhibitor

Wee1 and Chk1 inhibition

As Wee1 and Chk1 both regulates intra-S phase and G_2/M cell cycle checkpoints, agents targeting Chk1 and Wee1 are usually combined with DNA damaging agents, i.e. cytarabine or cisplatin[67]. RNA interference (RNAi)-induced silencing has been examined in a study that silenced 572 kinase for cytarabine sensitivity in acute myeloid leukemia (AML); Wee1 and Chk1 have been identified as important determinants of this cytarabine activity in the AML cells both *in vitro* and *ex vivo*[68]. The combined activity of Wee1 inhibitor AZD1775 and Chk1 inhibitor MK8776 shows better outcome than either of the agent alone[67]. While AZD1775 activates the Atr/Chk1 pathway in the AML cell lines indicated by Chk1 phosphorylation at Ser-317 and autophosphorylation at Ser-296, this is accompanied by increased phosphorylation of DNA double strand break marker H2AX. Addition of MK8776 results in increased AZD1775-induced phosphorylation of Chk1 on Ser-317 and H2AX phosphorylation on Ser-139. This enhanced efficacy is reflected in an increase in apoptosis[67]. Combined Wee1 and Chk1 inhibition has been proven useful in other studies as well.

Relapsed neuroblastoma is resistant to conventional chemotherapy and Wee1 has been found to be highly present these cancer type, particularly in the tumors that are rich in proto-oncogene *MYCN*[69]. Neuroblastoma shows sensitivity to single agent inhibition of AZD1775 and MK8776, however, inhibition of Wee1 and Chk1 both serves as a mechanism to sensitize these tumors to currently used chemotherapeutic agents. When combined with agents like gemcitabine (potently chemosensitized by Chk1 and Wee1 inhibition) and irinotecan (used clinically for treatment of relapsed neuroblastoma), AZD1775 and MK8776 could act synergistically by inducing double-strand breaks in DNA and forcing the cells to undergo mitotic catastrophe and subsequently, apoptosis. This dual inhibition therapy is also able to inhibit the growth of neuroblastoma xenografts *in vivo*[69].

The use of Chk1/Chk2 inhibitor PF-0047736 or AZD1775 in single agent gives limited toxicity on healthy cells using ex vivo therapeutic concentrations. Combination strategies allowed the use of sub-toxic concentrations of the two inhibitors which obtained same *in vitro* therapeutic effects like one of the two inhibitors in monotherapy. The effect of the combined treatment on primary acute lymphoblastic leukemic cells caused significant reduction of primary cell colonies[70]. Protein kinase B (Aky) signaling is found in active status in up to 70% of sporadic melanomas, but targeting this protein alone in preclinical models showed little efficacy[71]. Simultaneous treatment with Akt inhibitor AZD5363 and Wee1 inhibitor AZD1775 demonstrated >90% reduction in xenograft tumor development and showed no toxicity[71]. Whereas, sequential treatment was less effective and showed progressive toxicity[71]. This suggests selection of simultaneous treatment of drug combination rather than sequential approach for clinical evaluation.

Wee1 and Atr Inhibition

The majority of TNBC cells have p53 deficiency and are highly dependent on G2 checkpoint mediated by Wee1, which is why TNBC cells shows sensitivity towards AZD1775[72]. Study shows that Atr inhibitor AZD6738 can sensitize TNBC cells to the Wee1 inhibitor AZD1775[73]. Synergistic killing by Atr and Wee1 inhibitors is triggered by Wee1 inhibition–induced DNA damage during replication. Combined inhibition of Wee1 and Atr during replication leads to substantial cell killing, probably due to extensive genome damage that cannot be repaired before cells enter mitosis[40]. These coordinated effects of Wee1 and Atr on faithful cell cycle progression in cells with high baseline DNA damage opens a therapeutic window to lower the activity of these two kinases to levels that is lethal for cancer cells, but tolerable to normal ones[40]. Combination of AZD1775 and AZD6738 enhanced cisplastin-induced cell death in TNBC cells. Combination of Wee1 and Atr has led to tumor selective lethality of breast cancer model *in vivo*[40]. The combination treatment could inhibit the spread of tumor and prolonged survival with minimal side effects. Bone marrow and ileum like rapidly proliferating tissues did not show any sign of renewal defects. The study could also reveal that combined inhibition treatment caused cells to enter mitosis with unrepaired DNA leading towards mitotic catastrophe[40].

Polycomb protein EZH2 can act as a potent therapeutic target of Wee1 inhibitor combinations against TNBCs, as high expression of EZH2 may provide an environment for unscheduled mitosis and lead to apoptosis in these cancer types[74]. Drug resistance has been a common problem in case of frequently used anti-cancer drug cisplastin, which causes a major clinical complication. Wee1, Atr and Chk1 kinase inhibition can overcome cisplastin resistance, however, Wee1 inhibition is more potent in killing TNBC cells than the Atr or Chk1 inhibition and knockdown of Wee1 can confer sensitivity to cisplastin treatment[65]. Wee1 kinase inhibitor AZD1775 can also sensitize advanced squamous cell carcinoma of the head and neck (HNSCC) cells to cisplastin therapy both *in vitro* and *in vivo* based on their p53 mutational status[75].

Wee1 and mTOR Inhibition

Inhibition of the major signaling pathway of rat sarcoma viral oncogene homolog (RAS), i.e. P13K or Akt or mTOR has limited efficacy in RAS mutated cancer. This limitation of treatment efficacy is believed to be due to activation of some bypass signaling pathways which leads to failure in the efforts targeting multiple effectors in the cancers with RAS-positive mutation[76]. Combining the mTOR inhibitor with AZD1775 provided outstanding outcome with enhanced effect. Suppression of the phosphorylation of Akt and 4E-BP1 correlates with an increase in apoptosis in mutant RAS expressing cells by combined Wee1 plus mTOR inhibition[76]. Combination of AZD1775 and mTOR inhibitor ridaforolimus in 39 diverse cancer cell lines also showed synergistic treatment effect. The combined treatment could inhibit phosphor-Cdk1[77]. The synergy in outcome might be related to uncoupling the regulation of cell growth and division. In mutant *KRAS*-driven lung cancer, dual inhibition of Wee1 and mTOR showed synergistic effects both *in vitro* and *in vivo*[78]. Inhibition of mTOR after inducing DNA damage accumulation of mutant cells by AZD1775

causes compensation in activation of DNA repair which results in cytotoxic synergism of the tumor. In ovarian cancer, AZD1775 combined with mTOR inhibitor AZD2014 inhibited tumor growth both *in vitro* and *in vivo*. The synergy is independent of *TP53* mutation and this simultaneous inhibition causes massive DNA replication stress and DNA damage[60].

Combination of Wee1 and novel polymerase (PARP) inhibitor

Poly (ADP-ribose) polymerase (PARP) plays an important role in repairing single-strand DNA (ssDNA) breaks and PARP inhibition is now at the foreground of cancer therapeutics, particularly in cancers where there is defect in HR repair, such as *BRCA* mutations[82], [83]. With the inhibition of PARP activity, ssDNA lesions are converted to DNA double-strand breaks that creates dependency on the homologous recombination (HR) pathway for repair[82]. PARP inhibitors (PARPi) such as olaparib and rucaparib are approved to treat BRCA-defective advanced ovarian and prostate cancer patients, who have previously received chemotherapy[84]. Talazoparib and veliparib, two additional PARRis, are in advanced clinical trial[83]. Although, strategies to sensitize *BRCA* wild-type tumors to PARPi alone have achieved limited efficacy. Treatment with PARPi primarily results in partial tumor regression, rarely gives complete response and emergence of resistance within a year is one of the downside of the treatment[83]. Combination of PARPi with Wee1 inhibitor has gained interest in recent years. Wee1 inhibition can compromise HR by Cdk1 mediated phosphorylation of *BRCA1/2*[85]. Tumors exhibit sensitivity to drugs targeting PARP with enhanced efficacy of tumor growth repression in the combination therapy with Wee1 inhibitor.

Cancer exhibiting low expression in HR-related factors like ARID1A, 53BP1, RAD51, and CCDC6 are proved sensitive to PARP inhibitors[86]. Reportedly, Wee1 repression compromises HR function marked by reduced 53BP1 and RAD51 with subsequent accumulation of γH2AX. Cell lines and xenografts treated with AZD1775 demonstrate downregulation of 53BP1 and RAD51 and upregulation of γH2AX. These two factors are also decreased by olaparib in presence of AZD1775 in comparison to olaparib alone[86]. Other HR-related proteins, i.e. MRE11, ATM and NBS1 disrupts HR when lowered by modulation, to improve antitumor efficacy of PARPi. Combination of AZD1775 plus olaparib can also cause impairment in HR function by sensitizing these factors to olaparib in presence of AZD1775[86]. To exert anticancer effect, PARP inhibitors work by blocking SSB repair first, SSB is converted into DSB however; which can be repaired by HR feedback mechanism to impede PARPi's response in HR functional population. In gastric cancer (GC), AZD1775 in combined therapy with olaparib, caused impairment of HR and prevented DSB repair to augment DNA-damage mediated cytotoxicity in cancer cells[86]. Blockade of Wee1/Plk1 provided an HR defective environment, which enhanced function of olaparib and increased antitumor activity of olaparib plus AZD1775 combination (Figure 4).The figure summarizes that limited efficacy of olaparib alone in treatment of GC occurs by functional HR which causes conversion SSB into DSB, but the dual inhibitory effect impairs functionality of HR, promoting lethality of GC cells to PARP inhibition.

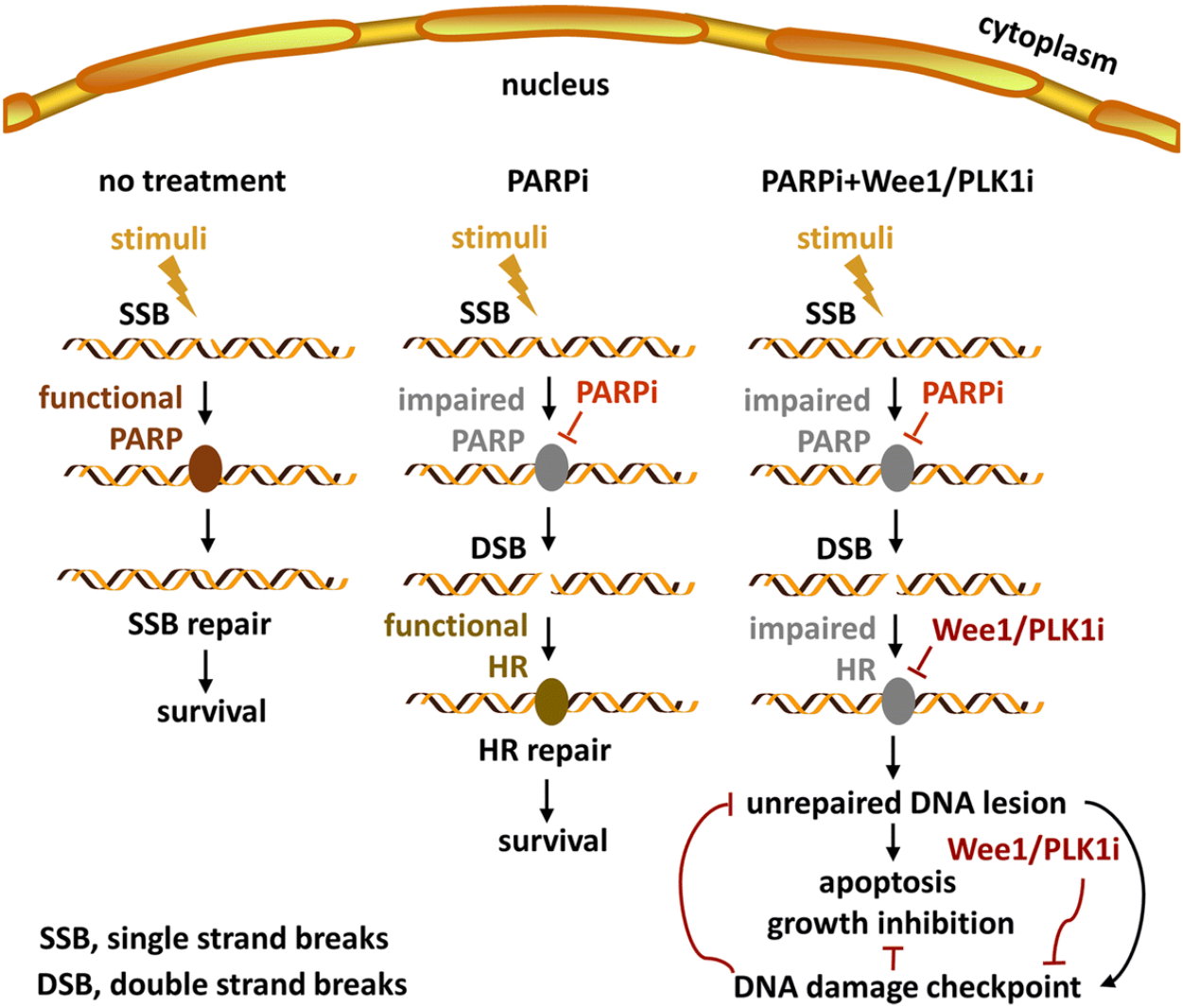

Figure 4. Illustration of enhanced anticancer activity in GC by PARP+Wee1/Plk1[86]

In pancreatic cancer, combined inhibition of Wee1 along with PARP inhibition produces remarkably more radiosensitization in the cancer cells that either Wee1 or PARP inhibition monotherapy[87]. AZD1775 inhibits HR repair and abrogates prolonged G2 checkpoint in combination with olaparib and radiation therapy. In addition to these effects, as Wee1 and PARP also interact to regulate replication stress, inhibiting them together causes PARP to mitigate the effect of replication stress induced by Wee1 inhibition[87]. In *KRAS* mutated lung cancer, combined olaparib and AZD1775 enhanced radiosensitization[88]. The strength of the combination strategy in KRAS-mutant lung cancer cells lies in the fact that enhanced radiosensitization by these two drugs cannot be rescued by addition of nucleosides, unlike radiosensitization by AZD1775 alone. Moreover, majority of SCLC patients acquire rapid chemo-resistance and thus results in disease progression. Tumor model generated from patient who has developed resistance against platinum

based therapy and is relatively insensitive to olaparib or AZD1775 alone, has shown durable response to AZD1775 plus olaparib combination treatment[38]. In AML and ALL cell lines and AML patients' samples, combination of olaparib and AZD1775 inhibits proliferation and enhances apoptosis. This combination can also significantly prolong survival of mice with murine AML[89]. In *TP53* mutated endometrium and ovarian cancer cell lines, Wee1 inhibition in combination with olaparib shows significant cell death in mitosis, as AZD1775 increase sensitivity of the cancer cells to olaparib[90].

The toxicity profile of Wee1 and PARP inhibition suggests replication stress in the concurrent administration of Wee1 inhibitor and PARPi. Concurrent therapy leading to weight loss in animal models and difficulty in finding effective doses in human clinical trials with less toxicity led to cessation of therapy[85]. The main reason behind this toxicity is because concurrent therapy with Wee1 and PARP inhibitors induces replication stress and DNA damage in normal cell culminating in cell death. Whereas, sequential PARP and Wee1 inhibition results in minimum increase in replication stress, DNA damage and cell death (Figure 5)[85]. High endogenous replication stress present in tumor cell, but absent in normal cells, justifies the tolerance of this combined inhibition in normal cells while maintaining treatment efficacy in tumors.

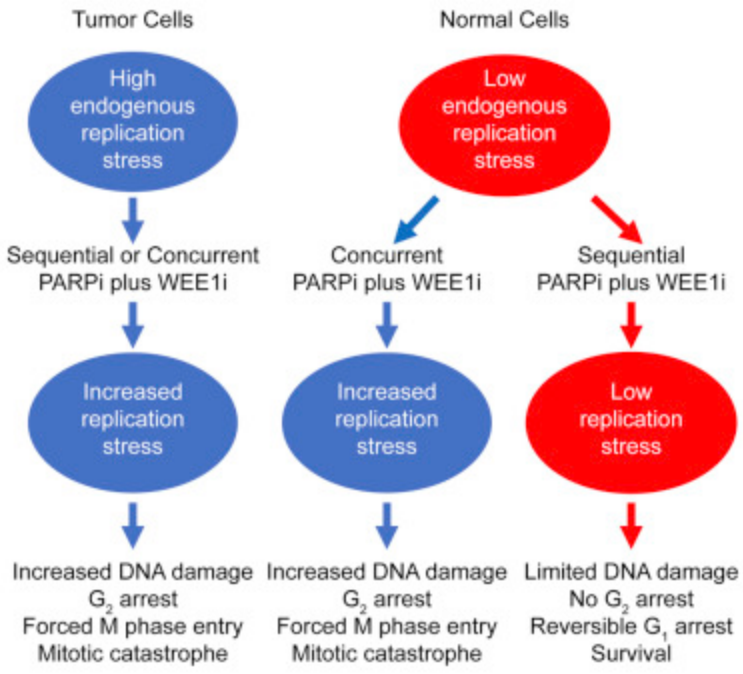

Figure 5. Enhanced tolerability to PARP and Wee1 inhibitors by sequential therapy[85]

The current clinical trial with PARPi and AZD1775 focuses mainly on the role of Wee1 in inducing G2 arrest, with the target of eliminating this arrest thus preventing DNA repair by HR restoration, in tumors treated with PAPRi. The clinical trial with this dual inhibition requires patients having

adequate bone marrow function as gastrointentinal toxicity and bone marrow suppression are some major side effects[83]. In summary, the gradually evolving combination therapy with PARPi and Wee1 inhibitor AZD1775 is a field rich in opportunity. Disruption of DNA damage checkpoint and DDR pathway increases DNA damage and apoptosis in cancer cells by the combination therapy both *in vitro* and *in vivo*. With a better understanding of PARP inhibitor's effect can extend its accessibility from the range of HR-deficient patients to HR-proficient patients, which can benefit patients in the coming years to implement cancer targeted therapy with this dual inhibition with much more efficacy.

Wee1 Inhibition in Combination with Other Targeted Molecules

As Wee1 is a client protein of molecular chaperone Hsp90, several studies have demonstrated that inhibition of Wee1 sensitized cervical and prostate cancer cells to the Hsp90 inhibitor and can promote binding to ATP-binding pocket of Hsp90, interfering with its function[25]. Wee inhibition can also sensitize tumor xenograft to Hsp90 inhibition[79]. Combined inhibition of Wee1 and Hsp70 thus can invoke intrinsic activation of apoptotic pathway. Wee1 inhibition can enhance caspase-dependent apoptosis in TNBC cell lines in combination with TNF-related apoptosis-inducing ligand (TRAIL)[80]. Chemical inhibition of Wee1 alone also upregulated transcription of TRAIL receptors in p53-independent fashion. AZD1775 displayed synergistic antitumor activity in lung cancer xenograft model *in vivo* when combined with Sirt1 inhibitor Ex527[81]. Cdk1, the main target of Wee1, plays a significant role in HR and double strand DNA repair, highlighting its potential to be targeted in combination of Wee1[25]. Chemical inhibition of Wee1 with forced activation of Cdk1, appears to impair HR. Cdk activity in low level is required for induction of HR repair, whereas, Cdk inhibition impairs the DDR. Wee1 inhibition causes hyperactivity of Cdk, which appears to fall in an outcome of comparable impairment of HR-DDR[25].

In summary, the potential of Wee1 inhibition in combination with chemotherapeutic agents, radioactive agents and other checkpoint kinase inhibitors can lead a new path to be tested in the clinical setting to be used as therapeutics in many of the cancer types.

Clinical Studies with Wee1 Inhibitors

On the basis of potential preclinical results with Wee1 inhibitor AZD1775, phase I and phase II studies are testing escalating single and also multiple doses of AZD1775 as monotherapy and in combination with cisplatin, gemcitabine or carboplatin in patients with advanced solid stage tumors and the results have shown promising outcomes.

Phase I clinical trials

A single agent phase I study with AZD1775 in patients with refractory solid tumor carrying *BRCA* mutations (with head and neck cancer and ovarian cancer) could give direct evidence of decrease in Wee1 kinase target pY15-Cdk levels in paired tumor biopsies[41]. An increase in γH2AX levels in paired tumor biopsies could also demonstrate the evidence of DNA damage response by the Wee1 inhibitor. Cytotoxicity studies could reveal common toxicities like diarrhea and

myelosppression[41]. Another study revealed the antitumor activity, safety and tolerability of AZD1775 in combination with gemcitabine, cisplatin and carboplatin and found AZD1775 to be well tolerated in general[91]. The oral administration of AZD1775 with the chemotherapeutic agents exceeded the threshold pharmacokinetic level of efficacy and provided evidence of Wee1 inhibition in combination with DNA damaging agents. The toxicity observed in AZD1775 single dose therapy remained consistent in the combination therapy with individual chemotherapeutic agents as well[91]. Neoadjuvant chemotherapy has extensively been studied in locally advanced HNSCC. The completed phase I clinical trial using AZD1775 with docetaxel and cisplatin in neoadjuvant setting in HNSCC patients could reveal a safe and tolerable triplet combination[92]. Acute toxicity with long-term postoperative radiotherapy (PORT) or postoperative chemoradiation (POCRT) has a considerable negative impact on cancer patients. A long-term treatment with AZD1775 in the neoadjuvant setting to downregulate the tumor proliferative activity through treatment in combination with cisplatin may decrease the extent of surgery, moreover can also reduce the need of PORCT[93]. This reduces the chance of developing relapsed disease within the area treated with high radiation dose. Phase I study with AZD1775 in head and neck cancer combined with chemoradiation enhanced the biological damage cause by cisplatin and radiation, with minimal toxicity[93]. A phase I dose escalation trial of AZD1775 combined with gemcitabine and radiation was conducted in patients with locally advanced pancreatic cancer who were previously untreated. Locally advanced pancreatic cancer when treated with chemotherapy alone, demonstrate less survival benefits in patients. When patients were treated with AZD1775 combined with gemcitabine and concurrent radiation therapy, favorable survival was observed which can be related to the sensitization of disease both locally and distantly to treatment by AZD1775[94].

Phase II clinical trials

The clinical trials with AZD1775 has successfully reached in phase II trials with some caner types. Phase II study of AZD1775 has been done in combination with carboplatin in patients with early resistant (<3 months) ovarian cancer. The resistance occurred after first line platinum based therapy and mutation in p53 was frequently observed in the patients. Some tumors were resensitized to therapy with carboplatin by abrogating G2 checkpoint using AZD1775. Alteration in gene groups like *TP53*, *RB1*, *BRCA1* and some oncogene induced replication stress genes (*KRAS* and *MYC*) were seen to be altered with this Wee1 inhibition[95]. Patients with ovarian, primary peritoneal and fallopian tube tumors with p53 mutational status receiving AZD1775 along with paclitaxel and carboplatin established tolerability to AZD1775 with this combination in phase II clinical trial. When comparison was made between AZD1775 plus paclitaxel and carboplatin and placebo (inert treatment with no therapeutic value) plus paclitaxel and carboplatin; AZD1775 combined with paclitaxel and carboplatin showed marked improvement in progression free survival (PFS) and fulfilled the criteria of PFS efficacy in accordance with clinical significance[96]. A phase II clinical trial with AZD1775 alone in patients with breast, ovarian, pancreatic and some other cancer types with *BRCA 1/2* mutated tumors previously treated with PARPi and platinum

based treatment showed modest anti-tumor activity of AZD1775 and the dose was well tolerated[97]. The study suggests a better outcome in patients with *BRCA 1/2* mutated tumors, who are less heavily pretreated.

Some phase II studies are currently underway with AZD1775: a phase II study is evaluating the safety and efficacy of olaparib monotherapy versus olaparib plus AZD1775 in patients with TNBC. In case of invasive TNBC, olaparib shows enhanced PFS in patients who are negative for proto-oncogene human epidermal growth factor receptor 2 (*HER2*), and have germline BRCA mutation. Olaparib with AZD1775 targets DNA damage repair and had synergistic antitumor effects compared to olaparib monotherapy in preclinical studies which has led to an ongoing trial[98]. Two randomized phase II clinical trials in *TP53* mutated patients with platinum resistant ovarian, primary peritoneal and fallopian tube cancer with AZD1775 plus gemcitabine, AZD1775 plus paclitaxel, AZD1775 plus carboplatin, and AZD1775 plus PLD (Pegylated liposomal doxorubicin) is ongoing, which are trying to evaluate the dosage and safety of the combined treatment and compare the efficacy of the combined treatments with the chemotherapeutic agents alone[99], [100].

Phase I and phase II clinical trials with Wee1 inhibitor has showed improved clinical response rates in lengthening progression-free survival and overall survival of patients. It has overcome the problem in patients who acquire rapid drug resistance. The patients who do not receive prior long course of traditional anticancer treatment regimes seem to show better outcome with the Wee1 inhibition in trials, however, this is not necessarily a case in all type of cancers. The status of DDR is being characterized across wide range of tumors and validation of potential biomarker is going on to reveal more important molecular insights about response in DNA damage. Oral administration of the combination therapies with Wee1 and other chemotherapeutic agents has been seen to be well tolerated by the patients with mild side effects, which supports the fact of Wee1 inhibitor being used as traditional anticancer therapeutic in near future.

Concluding Remarks

Wee1 kinase, as a gatekeeper of G2 checkpoint arrest, holds a great potential therapeutic approach in the battle against cancer. Wee1 is highly expressed in many cancer types and is associated with progression of tumor and poor prognosis. Wee1 inhibition can sensitize cancers that depend on functional G2/M checkpoint to DNA damaging therapies. In contrast to other kinase inhibitors that aims for the cell cycle arrest, Wee1 inhibition forces cells towards apoptosis from mitotic catastrophe. Wee1 inhibition, when combined with chemotherapeutic agents, may allow reduction in the amount of their dosage, thus can reduce cytotoxicity from those agents. It can sensitize tumors to other conventionally used therapies, which otherwise give very poor outcome. Wee1 inhibitor AZD1775 has been extensively studied in preclinical xenograft models as single agent and as sensitizer for chemotherapy or radiation. In most of the studies, AZD1775 has been used at a dose below maximum tolerated dose.

Wee1 inhibitor has been demonstrated to be well tolerated in the patients in advanced clinical trial with better survival. So far, the clinical studies with AZD1775 have been shown to have better outcome when combined with other DNA-damaging agents, however, the expected anti-cancer effect from the agent alone needs to be evaluated more in clinical studies. Another reason for trials with AZD1775 being administered in combination with other DNA damaging agents can e speculated form the concern that this combination therapy can mask the toxicities of AZD1775, as AZD1775 has showed single agent toxicities in cell systems[8]. Another concerned focus should be determining the off-target effect of the Wee1 inhibitor to improve the therapeutic index and cytotoxicity profile. The biomarker for Wee1 sensitivity at the tumor site is still not verified. It is needed to provide an accurate representation of Wee1 engagement in the sample tissue with less toxicity and maximal ease. The optimal timing for treatment with DNA damaging component and G2 abrogating component is still not addressed properly.

Resistance to Wee1 inhibitor has been seen to occur due to upregulation of kinases like Myt, AXL or MET, however, methods have been developed to overcome these resistance. Moreover, in most of the studies Wee1 inhibitor has generally been more effective in p53-deficient tumors, so the mutational status of *TP53* holds a great significance in Wee1 inhibition therapy. The Chk1 and Atr inhibitors can enhance the efficiency of Wee1 inhibition. Combining Wee1 inhibitor with PARPi is also a great therapeutic strategy. Taken together, the efficacy of treatment with Wee1 inhibitor depends on multiple factors.

Another important concern of Wee1 inhibitor is selection criteria of patients. Patients who had confirmed locally advanced or metastatic solid tumor, failed to respond to standard therapy or progressed disease in spite of standard therapy are good candidates for selection. Patients who had undergone limited amount of radiation therapy can provide better outcome. Difference in tumor response endpoint in different type of tumors by Wee1 inhibition can be of significant use to measure the duration of response by this inhibitor, as response varied considerably between different cell lines and in patients as well.

With support from the clinical studies that have already been done, it can be postulated that Wee1 inhibitors can improve the therapeutic approach that are currently being used for cancer patients. Therefore, on the basis of all the facts discussed above it can be concluded that, Wee1 inhibition is a feasible option for anti-cancer therapy and with the extensive studies effective Wee1 inhibitors will prove to be useful to advance the treatment in cancer.

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