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Biomarker-Guided Development of DNA Repair Inhibitors

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Abstract

Anti-cancer drugs targeting the DNA damage response (DDR) exploit genetic or functional defects in this pathway through synthetic lethal mechanisms. For example, defects in homologous recombination (HR) repair arise in cancer cells through inherited or acquired mutations in *BRCA1, BRCA2*, or other genes in the Fanconi Anemia/BRCA pathway, and these tumors have been shown to be particularly sensitive to inhibitors of the base excision repair (BER) protein poly (ADP-ribose) polymerase (PARP). Recent work has identified additional genomic and functional assays of DNA repair that provide new predictive and pharmacodynamic biomarkers for these targeted therapies. Here, we examine the development of selective agents targeting DNA repair, including PARP inhibitors, inhibitors of the DNA damage kinases ATR, CHK1, WEE1, and ATM, as well as inhibitors of classical non-homologous end-joining (cNHEJ) and alternative end-joining (Alt-EJ). We also review the biomarkers that guide the use of these agents and current clinical trials with these therapies.

eTOC Blurb

In recent years, there has been substantial progress in our understanding of DNA damage response (DDR) pathways which has led to the development of a multitude of DDR inhibitors. This review focuses on therapeutic strategies of utilizing DDR inhibitors and biomarkers that can guide their clinical development.

Keywords

PARP inhibitor; DNA repair; Cell cycle kinases; Polymerase theta

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INTRODUCTION

The maintenance of genomic stability requires a protective cellular response to DNA damaging agents. Genomic instability, which is a hallmark of cancer cells, results from a defect in this DNA Damage Response (DDR). The DDR pathway is an intricately regulated system designed to protect cells against acquired changes to the genome and has evolved to safeguard against intrinsic and extrinsic DNA damage. This pathway encompasses proteins that detect DNA damage, function in DNA repair pathways, and regulate the cell cycle. Oncologists have been therapeutically targeting the DDR pathway for decades with cytotoxic agents. Not surprisingly, many of the mechanisms of sensitivity and resistance to cytotoxic chemotherapy are governed by the DDR pathway. In recent years, new anti-cancer drugs have been generated that inhibit the regulatory pathways governing the DDR response (Table 1). This review will summarize progress made in targeting the DDR pathway and highlight efforts to rationally combine these drugs utilizing biomarkers that predict their sensitivity and resistance.

Molecular Mechanisms of DNA Damage Induced by Cytotoxic Chemotherapy and Radiation

The anti-cancer efficacy of cytotoxic chemotherapy and radiation is derived from their ability to damage DNA. Analysis of the molecular mechanism of how these treatments damage DNA, and are subsequently repaired, has increased our understanding of the DDR pathway and has exposed potential weakness that can be exploited with molecularly targeted therapies. For example, DNA damage caused by the alkylating chemotherapy temozolomide (TMZ), which is commonly used in glioblastoma and pancreatic neuroendocrine tumors, is repaired by MGMT (methylguanine methyltransferase). This finding provides a rationale for generating pharmacological inhibitors of MGMT that may further sensitize tumors to TMZ. To date, the clinical utilization of MGMT inhibitors, such as O6-benzylguanine, has been hampered by significant myelosuppression (Quinn et al., 2009).

Cisplatin mainly exerts damage on DNA by forming intrastrand crosslinks, with the formation of guanine-platinum-guanine and guanine-platinum-adenine adducts (Rocha et al., 2018). While approximately 90% of the crosslinks formed by cisplatin are intrastrand, rarely cisplatin can also generate guanine-platinum-guanine interstrand crosslinks (Rocha et al., 2018). Intrastrand DNA crosslinks are repaired by nucleotide excision repair (NER), a mechanism of single-strand DNA repair which is deficient in patients with xeroderma pigmentosum (Scharer, 2013). Consistent with this, deleterious mutations in an NER pathway helicase, ERRC2, are predictive of cisplatin sensitivity in bladder cancer patients (Van Allen et al., 2014). Repair of interstrand crosslinks, which can also be generated by mitomycin-C, requires both NER and homologous recombination (HR) (Damia and Broggini, 2019; Rocha et al., 2018).

Radiation is a particularly effective anti-cancer therapy because it can sever covalent bonds in the DNA phosphodiester backbone and generate double-strand DNA breaks (Toulany, 2019). Bleomycin, a chemotherapy used in the treatment of testicular cancer and Hodgkin's lymphoma, forms a complex with Fe2+ and O_2 and can thereby cleave DNA and generate double-strand DNA breaks (Chen et al., 2008). Topoisomerase I and II enzymes reduce

DNA supercoiling by transiently causing single or double-strand DNA breaks, respectively, to allow DNA unwinding. Topoisomerase I inhibitors, such as irinotecan and topotecan, stabilize the topoisomerase I-DNA cleavage complex and prevents the closure of the single-strand DNA break (Pommier et al., 2010). Likewise topoisomerase II inhibitors, like etoposide and doxorubicin, trap the topoisomerase II-DNA cleavage complex and thereby generate a double-strand DNA break (Pommier et al., 2010).

Regulation of Double-Strand Break Repair

The two canonical pathways of double-strand DNA break repair are homologous recombination (HR) and classical non-homologous end joining (cNHEJ). Homologous recombination is preferred because of its high fidelity in repairing the genome. In contrast, cNHEJ utilizes non-specific ligation to correct DNA breaks, resulting in error-prone repair (Chang et al., 2017). The process of V(D)J recombination in immune cells advantageously harnesses the high percentage of errors introduced by cNHEJ to generate a diverse array of antibodies and T cell receptors. cNHEJ leads to characteristic DNA damage lesions, such as large deletions, resulting in loss of heterozygosity (LOH) and telomeric allelic imbalance. This error pattern can generate a highly recognizable genomic scar in HR-deficient cells, thus yielding an opportunity for therapeutic biomarker development (Table 2). Mechanistically, a key step in cNHEJ is the binding of KU70 and KU80 to the DNA (Chang et al., 2017). This event activates DNA-PK which, in turn, activates a multi-protein complex of XRCC4, Artemis, and DNA Ligase. The importance of DNA-PK, a PI3K-related protein kinase, has driven the development of several DNA-PK inhibitors, including nedisertib (M3814), AZD7648, and M9831 (VX-984) (Blackford and Jackson, 2017) (Table 1).

Unlike cNHEJ, HR uses a template from a sister chromatid to achieve accurate DNA repair. While cNHEJ can occur at any time in the cell cycle, HR occurs only in the S and G2 phases. A trio of three proteins, MRE11–RAD50–NBS1 (also called the MRN complex), initiates the pathway by binding to double strand DNA breaks (Ranjha et al., 2018). The MRN complex, in concert with the CtIP endonuclease and BRCA1, mediates DNA end resection. In addition, the MRN complex activates ATM, which leads to the activation of BRCA1, BRCA2, and PALB2. Following this event, RAD51 loads at the sites of DNA damage and results in the formation of a nucleoprotein filament that then invades the sister chromatid to find homologous DNA sequences that serve as a template for the synthesis of new DNA. Inhibitors of ATM and RAD51 are currently under development.

The HR pathway is deficient in Fanconi Anemia (FA), a rare genetic disease leading to bone marrow failure and increased cancer risk. Tumor cells with a deficiency in the Fanconi Anemia/BRCA pathway are hypersensitive to chemotherapies that cause DNA interstrand crosslinks, such as mitomycin-C and cisplatin, and, in some cases, to PARP inhibitors(Table 2). Cells from FA patients also demonstrate chromosomal instability. Indeed, the *BRCA2* breast cancer susceptibility gene was previously mapped to the FANCD1 complementation group (Howlett et al., 2002), thereby establishing the so-called Fanconi Anemia/BRCA pathway.

PARP inhibitors capitalize on the HR deficiency of many cancers in part by inhibiting other DNA repair pathways such as BER. While PARP inhibitors target tumors with intrinsic HR

deficiency, additional therapies are under development to inhibit HR in tumors. For example, a first-in-class RAD51 inhibitor, CYT01B, can block HR and has demonstrated preclinical activity in cancer cells expressing activation-induced cytidine deaminase (AID), a protein promoting double-strand DNA breaks (Maclay et al., 2018). Importantly, CYT01B synergizes with PARP and ATR inhibitors in model systems (Maclay et al., 2018).

Unlike cNHEJ, HR requires the generation of a single-strand DNA 3' overhang at the end of a double-strand DNA break in a process known as end resection. The tumor suppressor p53-binding protein 1 (53BP1) and the shieldin complex, made up of the SHLD1, SHLD2, SHLD3, and REV7 proteins, blocks end resection (Noordermeer et al., 2018). By preventing formation of a single-strand DNA 3' overhang, these proteins promote cNHEJ. Importantly, regulation of this complex plays a key role in determining whether double-strand DNA breaks are repaired by HR or cNHEJ. BRCA1 and 53BP1 play opposing roles in determining whether HR or cNHEJ is utilized (Escribano-Diaz et al., 2013). BRCA1 stimulates the MRN complex to activate CtIP-mediated 5' to 3' end resection to promote HR (Makharashvili and Paull, 2015). Notably, in S phase, BRCA1 activation also leads to decreased amounts of 53BP1 on chromatin (Chapman et al., 2012; Escribano-Diaz et al., 2013; Isono et al., 2017). However, in G1, 53BP1 and its effector protein RIF1 prevent end resection by promoting the assembly of the shieldin complex and activating NHEJ (Chapman et al., 2012; Escribano-Diaz et al., 2013).

TRIP13 is an ATPase that inactivates the shieldin complex and activates the 5' to 3' resection of double-strand breaks, thereby promoting HR repair (Clairmont et al., 2020). Importantly, TRIP13 is amplified in many BRCAI-deficient tumors, and its upregulation may contribute to the intrinsic PARP inhibitor resistance of many of these tumors. Accordingly, a small molecule inhibitor of the ATPase domain of TRIP13 may provide a useful means of stabilizing the shieldin complex, promoting cNHEJ, blocking HR, and overcoming intrinsic PARP inhibitor resistance. Early studies suggest that development of TRIP13 inhibitors may be useful not only in the treatment of BRCAI-deficient tumor cells with intrinsic PARP inhibitor resistance, but also in tumors with acquired PARP inhibitor resistance (Clairmont et al., 2020).

The elucidation of a third mechanism of DSB repair has identified important new DDR drug targets. This mechanism, called alternative nonhomologous end-joining (Alt-EJ) or MMEJ, requires "microhomology" (i.e., homologous DNA sequences approximately 2-20 base pairs in length) at the DNA break points (Bennardo et al., 2008; Ranjha et al., 2018). Importantly, Alt-EJ is dependent on PARP1. Following binding of the double-strand break by the MRN complex and PARP1, Polymeraseθ (Polθ) mediates the repair of the break. While the alt-EJ repair system is used rarely in HR proficient tumors, HR deficiency results in increased dependency on alt-EJ (Ceccaldi et al., 2015). The dependence of alt-EJ on Polθ suggests that Polθ inhibitors may be effective for HR-deficient tumors.

The final type of DSB repair is termed single-strand annealing. Similar to Alt-EJ, single strand-annealing requires homologous DNA sites to catalyze the repair of the double-strand break (Bhargava et al., 2016). However, unlike Alt-EJ, single-strand annealing can take place over long stretches of DNA and lead to large deletions which can cause

intrachromosomal translocations. Mechanistically, single-strand annealing is inhibited by the RAD51 (Stark et al., 2004). Unlike Alt-EJ, which requires PARP and Polθ, single-strand annealing requires RAD52 to marshal the annealing of the homologous stretches of single-strand DNA (Bennardo et al., 2008; Grimme et al., 2010).

Mechanism of Action of PARP Inhibitors

Two seminal papers introduced the concept that PARP inhibitors have synthetic lethal interaction with HR-deficient tumors (Bryant et al., 2005; Farmer et al., 2005) (Figure 1). PARP1 binding to single-strand DNA breaks generated in BER forms a foundation of the synthetic lethal interaction with HR deficiency (De Vos et al., 2012; Strom et al., 2011). When BER cannot repair single-strand DNA breaks, the single-strand breaks ultimately become double-strand DNA breaks. While HR-proficient cells can rely on the HR repair to correct double-strand DNA breaks, HR-deficient cells are forced to rely on cNHEJ (McCabe et al., 2006). However, the error-prone nature of cNHEJ ultimately leads to genomic instability and cell death (Patel et al., 2011).

Preclinical and clinical studies of PARP inhibitors soon revealed additional mechanisms of PARP inhibitor activity. PARP inhibitors have been shown to “trap” the PARP enzyme at the site of DNA damage, therefore preventing essential cellular processes such as DNA repair and transcription. Furthermore, the trapped PARP-DNA complex is particularly lethal in HR-deficient cells. Multiple studies have demonstrated differential potency of different PARP inhibitors. In HR-deficient cell lines, the IC_{50} of talazoparib (5 nM) was significantly lower than that of olaparib (259 nM), rucaparib (609 nM), and veliparib (>10,000 nM) (Shen et al., 2013). One explanation for the increased potency of talazoparib is its strong PARP trapping activity, approximately 100-fold greater than that of other PARP inhibitors (Murai et al., 2014a). Similarly, the relatively weak PARP trapping of veliparib may account for its weaker anti-cancer activity, compared to other PARP inhibitors (Murai et al., 2012).

Clinical trials of PARP inhibitors have led to their approval by the United States Food and Drug Administration (FDA) in several indications, including ovarian, breast, and pancreatic cancer (Table 1)(Geenen et al., 2018; Golan et al., 2019; Konstantinopoulos and Matulonis, 2018). The anti-cancer efficacy and favorable side effect profile of PARP inhibitors has led to their rapid adoption into clinical practice. While myelosuppression can be a limiting toxicity, these orally administered drugs are generally well tolerated, with most patients reporting minimal symptomatic PARP-inhibitor induced toxicities.

Inhibition of Polθ-dependent alternative end-joining

In recent years, there has been growing appreciation for the role of the alt-EJ system in double-strand DNA repair (Bennardo et al., 2008; Wood and Doublié, 2016). Mice deficient in polymerase Polθ are viable and exhibit a mild phenotype (Shima et al., 2004). However, the addition of an HR-inactivating mutation in mice deficient in polymerase Polθ is embryonically lethal (Ceccaldi et al., 2015). Furthermore, shRNA depletion of Polθ, combined with pharmacological PARP inhibition, is cytotoxic in *in vitro* and *in vivo* models of HR-deficient ovarian cancer (Ceccaldi et al., 2015).

Polθ plays two critical roles in DNA double-strand repair (Black et al., 2016; Chan et al., 2010). The polymerase domain of Polθ functions in the alt-EJ pathway. In addition, a separate helicase domain of Polθ at the N-terminus has ATPase activity and has nearby RAD51 binding sites. The RAD51-binding activity of Polθ removes the RAD51 molecules that bind to DNA during HR-repair (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015). The removal of RAD51 is crucial since RAD51 accumulation can lead to cellular toxicity. Polθ inhibition may prevent removal of RAD51 foci and thus lead to toxicity in cells with HR alterations, thus providing a mechanism of action of Pole inhibitors (Ceccaldi et al., 2015). Polθ inhibition may be efficacious in both HR-deficient cells through inhibition of the alt-EJ pathway, as well as in cells with acquired PARP inhibitor resistance through disruption of HR restoration.

The potential synthetic lethality of Polθ inhibitors in HR-deficient tumors has created great interest in developing clinical grade Polθ inhibitors (Figure 1C). Whether Polθ inhibitors should be designed which target the C-terminal polymerase domain or the N-terminal ATPase domain remains an unresolved question. Recent evidence indicates that inhibiting the ATPase of Pole results in the accumulation of toxic RAD51 complexes at the sites of abortive HR repair, further suggesting a synthetic lethality mechanism resulting from the knockout of both HR and Pole (Ceccaldi et al., 2015).

Mechanisms of Resistance in HR-deficient Tumors

Cancer cells can develop acquired resistance to PARP inhibition through several mechanisms (Table 3). Similar to other systemic therapies, one mechanism of acquired resistance is through increasing the efflux of the PARP inhibitors out of the cell by upregulating drug-efflux transporters such as the multidrug resistance pumps (MDRs) (Rottenberg et al., 2008). Alterations in the function of the PARP1 protein, by *PARP1* mutations which reduce PARP1 binding affinity to DNA or disrupt PARylation, via disruption of PAR removal, also lead to acquired PARP inhibitor resistance (Gogola et al., 2018; Pettitt et al., 2018).

Cancer cells may also develop acquired resistance to PARP inhibitors by either restoring HR repair or by re-establishing replication fork stability (Konstantinopoulos et al., 2015; Ray Chaudhuri et al., 2016; Rondinelli et al., 2017b; Yazinski et al., 2017). Additionally, recent work has shown that these mechanisms may occur together (Yazinski et al., 2017). Reversion mutations in HR genes that restore function of the HR proteins have been observed in samples from patients who have developed acquired resistance to PARP inhibitors. Alternatively, increased expression of hypomorphic mutant proteins capable of RAD51 loading may also restore HR (Johnson et al., 2013).

Notably, HR deficiency has been shown to sensitize cancer cells to platinum-based chemotherapy, and several of the mechanisms that confer resistance to PARP inhibition have also been demonstrated in patients with acquired platinum chemotherapy resistance (Pishvaian et al., 2017; Sakai et al., 2008; Weigelt et al., 2017). Conversely, mechanisms of PARP inhibitor resistance and cisplatin resistance do not always track together (Johnson et al., 2016). For example, REV7 depletion will cause PARP inhibitor resistance by promoting DNA end resection but will also create an acquired vulnerability to platinum based on its

role in translesion synthesis in the Fanconi Anemia pathway (Niimi et al., 2014). Moreover, in ovarian cancer, responses to platinum-based chemotherapy have been observed after acquired PARP inhibitor resistance has manifested (Ang et al., 2013).

Predictive and Pharmacodynamic Biomarkers for DDR Inhibitor Drug Development

While PARP inhibitors have shown efficacy in HR-deficient breast and ovarian cancer, there is great need to discover additional biomarkers that can identify tumors susceptible to PARP inhibitors as well as other DDR inhibitors (Table 2). PARP inhibitors are well known to have activity in *BRCA1* and *BRCA2*-mutated tumors; however, PARP inhibitors also demonstrate activity in tumors with other HR mutations such as *PALB2, RAD51C*, and *RAD51D* (Chandran and Kennedy, 2018). In addition to mutational analysis, an analysis of BRCA1 methylation demonstrated that homozygous *BRCA1* promoter methylation is predictive of response to rucaparib in a cohort of 21 ovarian cancer patients on the ARIEL2 trial (Kondrashova et al., 2018; Swisher et al., 2017).

A genomic mutational signature, termed signature 3, is associated with homologous recombination (HR) repair deficiency (Alexandrov et al., 2013; Polak et al., 2017). While signature 3 shows strong concordance with known pathogenic biallelic mutations in HR genes, many patients display a mutational signature of HR deficiency but do not demonstrate a clear mutation in *BRCA1, BRCA2*, or *PALB2* (Polak et al., 2017), suggesting that additional alterations in the HR pathway can also underlie such a mutational signature. The requirement for whole-genome or whole-exome sequencing for identification of signature 3 in tumor samples has limited its widespread clinical utilization as biomarker; however, computational tools have recently been developed that allow detection of signature 3 using clinical-grade targeted sequencing panels (Gulhan et al., 2019). With this methodology, signature 3 was a powerful predictor of response to a niraparib/pembrolizumab combination in a molecularly unselected population of platinum-resistant ovarian cancer patients (Farkkila et al., 2020).

The possibility of acquired resistance to PARP inhibitors through genetic or non-genetic mechanisms highlights the need for functional biomarkers that can assess HR proficiency. For instance, an immunofluorescence-based RAD51 assay can interrogate HR proficiency in preclinical and clinical samples (Castroviejo-Bermejo et al., 2018; Hill et al., 2018; Mukhopadhyay et al., 2010; Naipal et al., 2014). Since the formation of RAD51 foci at sites of double-strand DNA damage is a critical step in the HR pathway, this assay differentiates between HR-proficient cancers, which form RAD51 foci, and HR-deficient cancers which are unable to form RAD51 foci. One methodology for this assay utilizes irradiated live tumor cells, and this was shown to be predictive of HR-deficient human breast cancer (Naipal et al., 2014). However, since live tumor cells are often not available in clinical specimens, there also has been efforts to perform the RAD51 assay on formalin fixed samples. Using formalin fixed samples, several groups have been able to demonstrate that the RAD51 assay can identify HR-deficient tumor cells that were appropriately PARP inhibitor sensitive (Castroviejo-Bermejo et al., 2018; Hill et al., 2018; Mukhopadhyay et al., 2010).

Platinum sensitivity is increasingly utilized as a biomarker for PARP inhibitor sensitivity in clinical trials (Table 2). The initial correlation of platinum sensitivity with PARP inhibitor sensitivity was observed in ovarian cancer. One study found a marked difference in olaparib sensitivity in platinum-sensitive and -resistant ovarian cancer patients (Fong et al., 2010). In pancreatic cancer, a similar trend has been observed. In a phase 2 trial of *BRCA*-deficient pancreatic cancer patients, none of the responding patients had previously progressed on platinum-based chemotherapy (Shroff et al., 2018). Building on this observation, the phase 3 POLO trial evaluated the efficacy of olaparib maintenance therapy following platinum-based chemotherapy in pancreatic cancer patients with *BRCA1 and BRCA2* germline mutations. This study was limited to patients who were platinum-sensitive, defined as not having disease progression over at least 16 weeks of platinum-containing chemotherapy. Compared to the patients randomized to placebo, the patients treated with maintenance olaparib had a 3.6-month increase in progression-free survival (PFS) (Golan et al., 2019). This study led to the FDA approval of olaparib maintenance therapy in pancreatic cancer patients with *BRCA1* and *BRCA2* germline mutations.

There has been great interest in developing integrative genomic assays that predict HR deficiency (Lord and Ashworth, 2016). Myriad Genetics developed a homologous recombination deficiency (HRD) assay that identifies HR-deficient tumors based on characteristic genomic findings of these tumors, including Loss of Heterozygosity (LOH), Telomeric Allelic Imbalance (TAI), and Large-scale State Transitions (LST). Another genomic assay, the Foundation Medicine T5 NGS assay, assesses the mutational status of 30 HR genes and also calculates the percentage of genomic LOH (Frampton et al., 2013). Both the Myriad HRD assay and the Foundation Medicine LOH assay have been validated by their ability to predict PARP inhibitor sensitivity in large phase 3 clinical trials in ovarian cancer of niraparib and rucaparib, respectively (Coleman et al., 2017; González-Martín et al., 2019; Swisher et al., 2017). However, while both the Myriad HRD assay and Foundation Medicine LOH assay are predictive of PARP inhibitor sensitivity, they failed to identify all of the platinum-sensitive patients who benefitted from PARP inhibition (Coleman et al., 2017; González-Martín et al., 2019; Swisher et al., 2017). This finding highlights the value of using platinum sensitivity as a surrogate biomarker for PARP inhibitor responsiveness in ovarian cancer.

Schlafen 11 (SLFN11) has emerged as a biomarker of PARP inhibitor sensitivity and resistance. In screens of cancer cell lines for genes predictive of cytotoxic chemotherapy sensitivity, a low level of SLNF11 was a biomarker of resistance to DNA damaging cytotoxic chemotherapies, including topoisomerase inhibitors, platinum chemotherapies, and alkylating agents (Barretina et al., 2012; Zoppoli et al., 2012). Subsequent studies demonstrated that low levels of SLFN11 are also associated with resistance to PARP inhibitors (Murai et al., 2016). While the function of SLFN11 is unknown, a recent study demonstrated that SLFN11 decreases protein synthesis of DDR proteins, such as ATR and ATM (Li et al., 2018). Consistent with this observation, PARP inhibitor resistance caused by low SNLF11 levels, can be overcome by combined inhibition of ATR and PARP (Murai et al., 2016). This has led to a hypothesis that SLNF11 promotes apoptosis in cells under extreme replicative stress, such as that conferred by viral infection or some cancers (Murai et al., 2019).

In addition to predictive biomarkers, the process of DDR drug development also requires pharmacodynamic (PD) biomarkers, which establish that the DDR inhibitor is adequately hitting the target (Table 2). Given their role in repairing DNA damage, increased γH2AX is a universal PD biomarker shared among all DDR repair inhibitors. In addition, PARP or POLθ inhibition results in increased levels of RAD51. A combination of a PARP inhibitor plus a POLθ inhibitor further increases RAD51 foci to toxic levels, further establishing the mechanism of action of POL0 inhibitors (Ceccaldi et al., 2015).

Recent work has demonstrated that mutations in the metabolic enzymes isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) increase the sensitivity of tumors to PARP inhibition (Molenaar et al., 2018; Sulkowski et al., 2017). A possible mechanism for this is that 2-Hydroxyglutarate (2HG), an oncometabolite produced by mutated IDH1 and IDH2, suppresses HR (Sulkowski et al., 2017). A clinical trial testing whether PARP inhibitor monotherapy has efficacy in IDH1 and IDH2 mutated tumors is currently underway (NCT03212274).

ATR, CHK1, and WEE1 inhibition in HR-deficient tumors

DNA damage-induced checkpoints facilitate the coordination between the DNA damage response and cell cycle control and allow ample time for repair and prevention of the permanent DNA damage produced by replication and mitosis. Two of the PI3K-related protein kinases (PIKKs), ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR), occupy a central role in signaling DNA damage to cell cycle checkpoints and DNA repair pathways (Curtin, 2012). Both checkpoint proteins play a critical role in maintaining the integrity of DNA. ATR homozygous knockout mice are embryonically lethal, while heterozygous ATR knockout mice ($ATR^{-/+}$) are viable but have increased cancer incidence (Brown and Baltimore, 2000). Conversely, while mice with a homozygous deficiency in ATM are not embryonically lethal, they do demonstrate a markedly increased sensitivity to double-strand DNA breaks caused by y-irradiation (Xu and Baltimore, 1996). In response to y-irradiation, primary cells from ATM-deficient mice did not undergo cell cycle arrest and fail to activate p53 (Xu and Baltimore, 1996).

ATM is primarily activated by double-strand DNA breaks. Activation of ATM leads to initiation of the HR-pathway machinery through the activation of BRCA1, BRCA2, and PALB2 and development of RAD51 foci. In addition to activating HR, ATM also activates CHK2 which in turn activates p53. While ATM is activated by double-strand DNA breaks, ATR is primarily activated by stalling or collapse of DNA replication forks, leading to the accumulation of ssDNA and replicative stress. When a replication fork becomes unstable, because of factors such as DNA damage or a lack of deoxyribonucleotide triphosphates (dNTPs), ssDNA becomes coated with phosphorylated-RPA (pRPA) (Dobbelstein and Sorensen, 2015). The presence of pRPA leads to the activation of the regulatory kinases ATR and its downstream effector CHK1, which govern the cellular response to replicative stress. In addition, activation of ATR/CHK1 leads to the activation of WEE1 and inhibition of CDC25A and CDC25B (Otto and Sicinski, 2017). In this way, activation of the ATR/CHK1/WEE1 pathway compensates for replication stress, resulting in prolongation of the S/G2 phase of the cell cycle (Dobbelstein and Sorensen, 2015).

The critical DDR functions of the ATM and ATR pathways create an opportunity for synthetic lethality. Preclinical investigation has demonstrated that *in vitro* models of *ATM-mutated* gastric cancer and chronic lymphocytic leukemia are very sensitive ATR inhibition (Kwok et al., 2016; Min et al., 2017). Accordingly, a phase 1 trial of the ATR inhibitor BAY1895344 showed partial responses in patients whose tumors lacked evidence of ATM protein expression (De Bono et al., 2019). Evaluation of ATM deficiency using immunohistochemistry enables the most comprehensive assessment of ATM expression at the protein level; however, genomic analysis to identify ATM mutations is a robust alternative. Moreover, improved methodologies are needed to identify and distinguish ATM biallelic or dominant-negative mutations.

Acquired resistance to PARP inhibitors remains an unsolved clinical problem. Since the primary mechanisms of PARP inhibitor resistance are 1) the restoration of HR repair and 2) the stabilization of the replication fork, new pharmacological strategies are needed to reverse these processes and to restore PARP inhibitor sensitivity. Inhibition of the ATR-CHK1-WEE1 signaling cascade is likely to prove useful in the treatment of PARP inhibitor resistance (Figure 2). PARP inhibitor treatment results in replicative stress and activation of the ATR/CHK1 pathway (Kim et al., 2017). PARP inhibitor-resistant tumors are hyper-dependent on the ATR-CHK1-WEE1 pathway, and subsequent inhibition of this regulatory pathway may restore PARP inhibitor sensitivity.

Indeed, ATR inhibition can re-sensitize *BRCA1*-mutated ovarian cancer models to PARP inhibition by reversal of HR and through fork destabilization and blockage of BRCA1-independent RAD51 loading (Yazinski et al., 2017). Similarly, the CHK1 inhibitor prexasertib (LY2606368) overcomes PARP inhibitor resistance in ovarian cancer preclinical models (Parmar et al., 2019). Mechanistically, prexasertib induced cytotoxicity by converting stable replication forks to unstable replications forks (Parmar et al., 2019). Additionally, CHK1 phosphorylates RAD51, an event required for RAD51 nucleofilament formation. CHK1 inhibition therefore can reduce HR and destabilize replication forks (Sorensen et al., 2005).

Early treatment of a tumor with a PARP inhibitor and an ATR-CHK1-WEE1 pathway inhibitor may result in even stronger synergy. For instance, the concurrent exposure to an ATR inhibitor may prevent the acquisition of PARP inhibitor resistance in the first place. Additionally, because tumors are often polyclonal, some clones may be more sensitive to a PARP inhibitor while others are more sensitive to an ATR inhibitor, providing an additional incentive for concurrent upfront treatment with both drugs. Consistent with these findings, ATR or CHK1 inhibitors can synergistically increase cancer cell apoptosis in PDX models of BRCA2-deficient ovarian cancer (Kim et al., 2017). Interestingly, combined ATR and PARP inhibition leads to cytotoxicity by causing premature entry into mitosis (Schoonen et al., 2019).

Inhibition of the ATR-CHK1-WEE1 pathway is complicated by induction of myelosuppression. In phase 1 trials, myelosuppression was the dose limiting toxicity of the ATR inhibitor BAY1895344, the CHK1 inhibitors GDC-0575, and the WEE1 inhibitor adavosertib (De Bono et al., 2019; Do et al., 2015; Italiano et al., 2018; Plummer et al.,

2019). A prime example of this challenge was the clinical development of the CHK1i prexasertib. After demonstrating initial encouraging results in ovarian and advanced squamous cell cancers, later phase trials faltered in part because of the treatment limitations of myelosuppression (Lee et al., 2018). Next generation CHK1 inhibitors, such as SRA737 (CCT245737), may mitigate the myelosuppression of other CHK1 inhibitors by decreasing simultaneous inhibition of CHK2 (Walton et al., 2016).

Therapeutic Strategies Designed to Increase Replicative Stress

Although some cancers have an inherent degree of endogenous replicative stress, agents that further increase replicative stress provide a novel treatment strategy. Topoisomerase I and II inhibitors generate replicative stress by preventing the repair of topoisomerase-generated DNA breaks and trapping the topoisomerase enzyme on DNA (Canela et al., 2019; Ubhi and Brown, 2019). This trapping blocks replication forks from proceeding. Furthermore, under normal circumstances, the PARP protein binds to topoisomerase I generated single-strand DNA breaks to stimulate the recruitment of DNA repair proteins such as tyrosyl-DNA phosphodiesterase 1 (TDP1) and XRCC1 (Das et al., 2014; Zhang et al., 2011). Not surprisingly, preclinical data has suggested that topoisomerase I inhibitors and PARP inhibitors are synergistic. Accordingly, preliminary signs of anti-cancer activity were observed in a phase 1 trial of the topoisomerase I inhibitor irinotecan and the PARP inhibitor veliparib (LoRusso et al., 2016; Zhang et al., 2011). Similarly, when compared to irinotecan monotherapy, combining irinotecan and ATR inhibition increases DNA damage and cancer cell death in *in vitro* and *in vivo* models (Josse et al., 2014). A phase 1 trial combining topotecan and the M6620 ATR inhibitor demonstrated preliminary signs of clinical benefit in several platinum-resistant small cell lung cancer patients (Thomas et al., 2018). Pharmacodynamic studies on this trial suggested that the topotecan/ATR inhibitor combination can increase the number of double-strand DNA breaks (Thomas et al., 2018).

Another widely recognized means of increasing replicative stress is to create conditions that decrease the cellular concentration of dNTPs, by using drugs such as hydroxyurea and gemcitabine (Ubhi and Brown, 2019). Depriving growing replication forks of dNTPs deprives DNA of its necessary building blocks, resulting in substantial replicative stress. Gemcitabine, a pyrimidine nucleoside analog commonly used for pancreatic and ovarian cancer, exerts its cytotoxicity through incorporation of its product dFdCTP into DNA, thus resulting in replication termination (Brunton et al., 2011). Unlike the pyrimidine analog cytarabine, gemcitabine also targets cancer cells by irreversibly inhibiting ribonucleotide reductase (Brunton et al., 2011; Moore and Cohen, 1967). Purine nucleotide analogs, such as clofarabine, fludarabine, and cladribine, are reversible inhibitors of ribonucleotide reductase (Tsesmetzis et al., 2018). The inhibition of ribonucleotide reductase, an enzyme that generates dNTPs, results in decreased dNTP concentrations and creates replicative stress (Ubhi and Brown, 2019). Pretreatment of tumor cells with gemcitabine stalls replication forks and activates the ATR-CHK1-WEE1 compensatory pathway, thereby stabilizing these stalled forks (Fordham et al., 2018; Koh et al., 2018). Accordingly, sequential treatment with gemcitabine, followed by an inhibitor of the ATR-CHK1-WEE1 pathway, provides a potent anticancer drug combination.

Clinical trials of CHK1 inhibitors have entailed a strategy of combining gemcitabine with CHK1 inhibition (Table 4)(Xiao et al., 2013). Like most therapies that increase replicative stress, a challenge is that these regimens could be too myelosuppressive. However, the high degree of synergy between gemcitabine and CHK1 inhibitors suggests that CHK1 inhibitors may be combined with substantially lower doses of gemcitabine than are typically used clinically. Through this strategy, low-dose gemcitabine generates replicative stress, creates a state dependent on the ATR-CHK1-WEE1 pathway, and primes cancer cells for the use of these kinase inhibitors. An additional advantage of this strategy is that low doses of gemcitabine avoid the myelosuppression that are induced by standard clinical doses. Recent phase 1 studies demonstrate that low doses of gemcitabine at 250 mg/m^2 on days 1, 8, and 15 of a 28-day cycle are able to be safely administered with the CHK1 inhibitor SRA737 (Banerji et al., 2019). Despite the low dose of gemcitabine, confirmed partial responses were observed in anogenital and ovarian cancers (Banerji et al., 2019). Notably, partial responses were not observed in a separate trial testing single-agent SRA737 (Plummer et al., 2019).

ATR inhibitors can also counteract replicative stress, suggesting that combining ATR inhibition with gemcitabine may also be an effective treatment strategy. In response to replicative stress, ATR functions to stabilize replication forks and increases RRM2 levels, thereby ensuring adequate levels of dNTPs (Buisson et al., 2015). Hence, ATR inhibition can prevent compensatory increases in RRM2 after its suppression by gemcitabine. The therapeutic effectiveness of combining gemcitabine with ATR inhibition has been shown in preclinical models of multiple tumor types (Fordham et al., 2018; Hall et al., 2014). In a panel of pancreatic cancer cells, the ATR inhibitor ceralasertib (AZD6738) prevented gemcitabine-induced CHK1 phosphorylation, intra-S-phase checkpoint activation, and compensatory RRM2 re-accumulation (Wallez et al., 2018). The combination of gemcitabine and ceralasertib had significant anti-tumor activity in the K8484 pancreatic cancer cell line murine allograft model (Wallez et al., 2018). By blocking the ability of the cancer cell to compensate for gemcitabine-induced replicative stress, ATR inhibitor treatment markedly increases biomarkers of replicative stress (pRPA) and DNA damage (γH2AX).

A randomized phase 2 clinical trial of the ATR inhibitor M6620 (VX-970) combined with gemcitabine, compared with single agent gemcitabine in platinum-resistant ovarian cancer was recently completed (Konstantinopoulos et al., 2020). Combination therapy was well tolerated. Compared to single agent gemcitabine, the gemcitabine/M6620 combination had a statistically-significant 8 week increase in progression-free survival (PFS) [HR 0.57, p = 0.044). A confirmatory phase 3 randomized study is being planned (Konstantinopoulos et al., 2020).

A key clinical question is the differential activity of ATR, CHK1, and WEE1 inhibitors. While the precise mechanistic differences among these DDR drugs is under investigation, some clear distinctions are emerging. First, since ATR signals at the beginning of the DDR damage pathway, alternative pathways to activate CHK1 exist; thus, ATR inhibition appears less potent as a monotherapy but may be more suitable as a partner in combinatorial regimens (Buisson et al., 2015; Young et al., 2019).

Furthermore, in addition to ATR activating CHK1, DNA-PK has also been shown to activate CHK1 (Buisson et al., 2015). CHK1's two upstream signaling inputs may distinguish the anti-tumor activity of ATR and CHK1 inhibitors. The WEE1 kinase is furthest downstream in the pathway, Intriguingly a study in diffuse large B cell lymphoma suggested that it had increased monotherapy potency compared to single agent ATR inhibition (Young et al., 2019). Further differences between WEE1 inhibition and ATR/CHK1 inhibition include the stage of the cell cycle where they exert their effect. ATR/CHK1 inhibitors interfere with the compensatory mechanisms which regulate S phase, while WEE1 inhibitors release cells into mitosis prematurely (Koh et al., 2018; Young et al., 2019). Finally, while the major toxicity of ATR, CHK1, and WEE1 inhibitors has been myelosuppression, a first generation CHK1 inhibitor, AZD7765, also caused cardiotoxicity, leading to the discontinuation of its development (Sausville et al., 2014).

Given the potential importance of replicative stress in drug efficacy, it is critical to develop biomarkers that can assess replication stress. pRPA and pKAP1, which are both downstream substrates of ATR, have been used as immunohistochemical markers of replicative stress (Hill et al., 2018; Zellweger et al., 2015). A more rigorous approach of evaluating replicative stress is the assessment of replication fork stability using a DNA fiber assay. DNA fiber assays assess replication fork stability by consecutively pulsing live cells with two labeled thymidine analogs, typically 5-iodo-2'-deoxyuridine (IdU) and 5-chloro-2'-deoxyuridine (CldU) (Hill et al., 2018; Quinet et al., 2017). During this assay, the cells are treated with a fork stalling drug, such as hydroxyurea, either after or between the thymidine analog pulses. If incorporation of the two labeled thymidine analogs is equivalent, this indicates the replication fork was stable. However, if there is less incorporation of the second thymidine analog, this result suggests the replication forks containing the second thymidine analog were degraded and hence indicates replication fork instability. The predictive potential of this approach was shown in a recent study of ovarian cancer organoids demonstrating that unstable replication forks correlate with sensitivity to CHK1i and ATRi (Hill et al., 2018). A major shortcoming of DNA fiber assays, especially in clinical settings, is the need for live tumor cells. A more general strategy for assessing whether an experimental drug or drug combination increases replicative stress is to utilize pre-treatment and on-treatment tumor biopsies. Immunohistochemical analysis of these biopsies can assess whether there are increasing levels of pRPA and pKAP1, while organoid cultures generated from the biopsies can be used to perform DNA fiber assays to explore if there is an increase in replication fork instability.

Combination of PARP inhibitors and Cytotoxic Agents

Cytotoxic chemotherapy agents that generate DNA damage may be effective combination partners for PARP inhibitors. Given the mechanism of action of topoisomerase inhibitors and TMZ, combinations of these drugs with PARP inhibitors have been extensively investigated. Topoisomerase I enzymes generate a topoisomerase I cleavage complex which is stabilized by the PARP1 protein (Thomas and Pommier, 2019). This mechanism provides a rationale for a combination of a PARP inhibitor plus a topoisomerase I inhibitor. Similarly, TMZ-induced DNA damage is typically repaired by BER, and the PARP1 protein typically

binds to the single-strand DNA generated by this reaction (Murai et al., 2014b). This mechanism suggests that TMZ and PARP inhibitors can also be synergistically combined.

Given the potential synergy of cytotoxic chemotherapy and PARP inhibitors, multiple clinical trials have been conducted exploring these combinations. However, when PARP inhibitors are combined with chemotherapy, achieving full dose chemotherapy has been challenging becaus0e of overlapping myelosuppression (Rajan et al., 2012). While the PARP inhibitor and cytotoxic chemotherapy combination provides enhanced tumoricidal activity, it also risks causing increased normal tissue toxicity. Therefore, the utility of a DDR inhibitor combined with cytotoxic chemotherapy requires establishment of a therapeutic index. If the DDR agent has the same activity in normal tissue and cancer tissue, then no advantage can be achieved. In general, cancer cells exhibit a greater baseline deficiency in the DDR and accumulate more damage than normal cells (Mahamud et al., 2017), and these differences can be leveraged to gain a therapeutic index. Accordingly, profiling DDR defects in specific cancers may guide the combined use of a DDR targeted agent with a cytotoxic agent.

Given the difficulty with myelosuppression, recent trials have explored different dosing strategies. Farago and colleagues found that limiting the administration of olaparib and TMZ 75 mg/m2 daily to the first 7 days of each 21-day cycle was feasible and safe (Farago et al., 2019). Encouragingly, in platinum-sensitive small cell lung cancer patients, the objective response rate to this combination was 47% and the median progression-free survival was 4.5 months. Another strategy being explored involves combining low dose TMZ with PARP inhibition (Stradella et al., 2019).

Combination of DDR Inhibitors with Immunotherapy

A major limitation of DDR inhibitors is the development of acquired resistance. To increase the durability of responses, there is substantial interest in combining DDR inhibitors with immunotherapy agents. The success of PD1 inhibition in cancers with mismatch repair deficiency is proof of principle that neoantigens generated by faulty DNA repair systems can drive an immune response (Le et al., 2017). However, unlike mismatch repair deficient tumors, many tumors do not have a sufficient neoantigen load to drive the immune response. While neoantigen generation by inhibiting DNA repair proteins may be insufficient to drive an immune response, other interactions between DNA repair proteins and the immunoregulators have the potential to catalyze an anti-tumor immune response (Mouw and D'Andrea, 2018). Double-strand DNA breaks can stimulate PD-L1 upregulation, through ATM/ATR/CHK1 signaling, in cancer cells (Sato et al., 2017). Similarly, PARP inhibitor treatment, perhaps through the introduction of DNA damage, also increases PD-L1 expression in breast cancer cells (Jiao et al., 2017). While DNA damage can upregulate PD-L1, the presence of cytoplasmic DNA also stimulates the cyclic GMP–AMP synthase (cGAS)/stimulator of interferon genes (STING) pathway. The cGAS/STING pathway is a component of the innate immune system, a system which evolved to prevent the introduction of viral DNA into the cell. Its activation causes proinflammatory cytokine secretion, such as type 1 interferon (Kwon and Bakhoum, 2019). In an *in vivo* model of BRCA1-deficient triple-negative breast cancer, PARP inhibitor mediated DNA damage and activated the

cGAS/STING pathway, leading to intratumoral CD8 T cell infiltration (Pantelidou et al., 2019).

Multiple clinical trials are underway that are combining DDR inhibitors with PD1 inhibitors. Among DDR inhibitor/immunotherapy combination trials, PARP inhibitors and PD1 inhibitors are the furthest in clinical development. Multiple single arm trials have shown, at best, modest anti-tumor activity. Randomized trials are needed to confirm whether the efficacy of these combinations is superior to PD1 monotherapy (Konstantinopoulos et al., 2019; Vinayak et al., 2019). Given the complexity of interpreting the efficacy of the DDR inhibitor/immunotherapy combination trials, biomarkers for predicting response are being developed. In addition to signature 3 as a biomarker of response in the aforementioned trial of combined niraparib and pembrolizumab in platinum-resistant ovarian cancer, a nanostring-based assay examining interferon gene expression was also predictive of response (Farkkila et al., 2020). Finally, immunocompetent models will be required to preclinically assess combinations of DDR inhibitors with immunotherapies, including appropriate genetically-engineered mouse models, syngeneic models, or humanized mice harboring human cell line xenografts.

CONCLUSIONS

The success of PARP inhibitors in HR-deficient ovarian, breast, and pancreatic cancers highlights the potential of DDR inhibitors. Recently, several other DDR inhibitors, including those targeting ATM, ATR, CHK1, DNA-PK and WEE1 have entered into clinical trials. While many of trials with these agents have been monotherapy trials, there is an increasing number of clinical trials utilizing combinations of these agents.

While there is a temptation to combine drugs in trials because they are available, it is essential that a strong preclinical background provide the rationale of any proposed clinical study. Given the molecular heterogeneity of cancer patients and the risk of mounting toxicities from DDR inhibitor combinations, great care is required to define the optimal clinical setting, dose intensity, and schedule of these agents. Predictive biomarkers, which have been rigorously validated preclinically, can be utilized in DDR inhibitor clinical trial design to define the most suitable patient population. Furthermore, to ensure that clinical studies generate useful mechanistic observations, clinical trials of DDR combinations should incorporate pharmacodynamic biomarkers that can molecularly interrogate whether a drug hits the desired target or pathway. In addition, molecular understanding of the mechanisms of resistance to DDR inhibitors will spur development of strategies that can delay, or reverse, acquired resistance to these agents and optimize clinical benefit from DDR-directed therapies.

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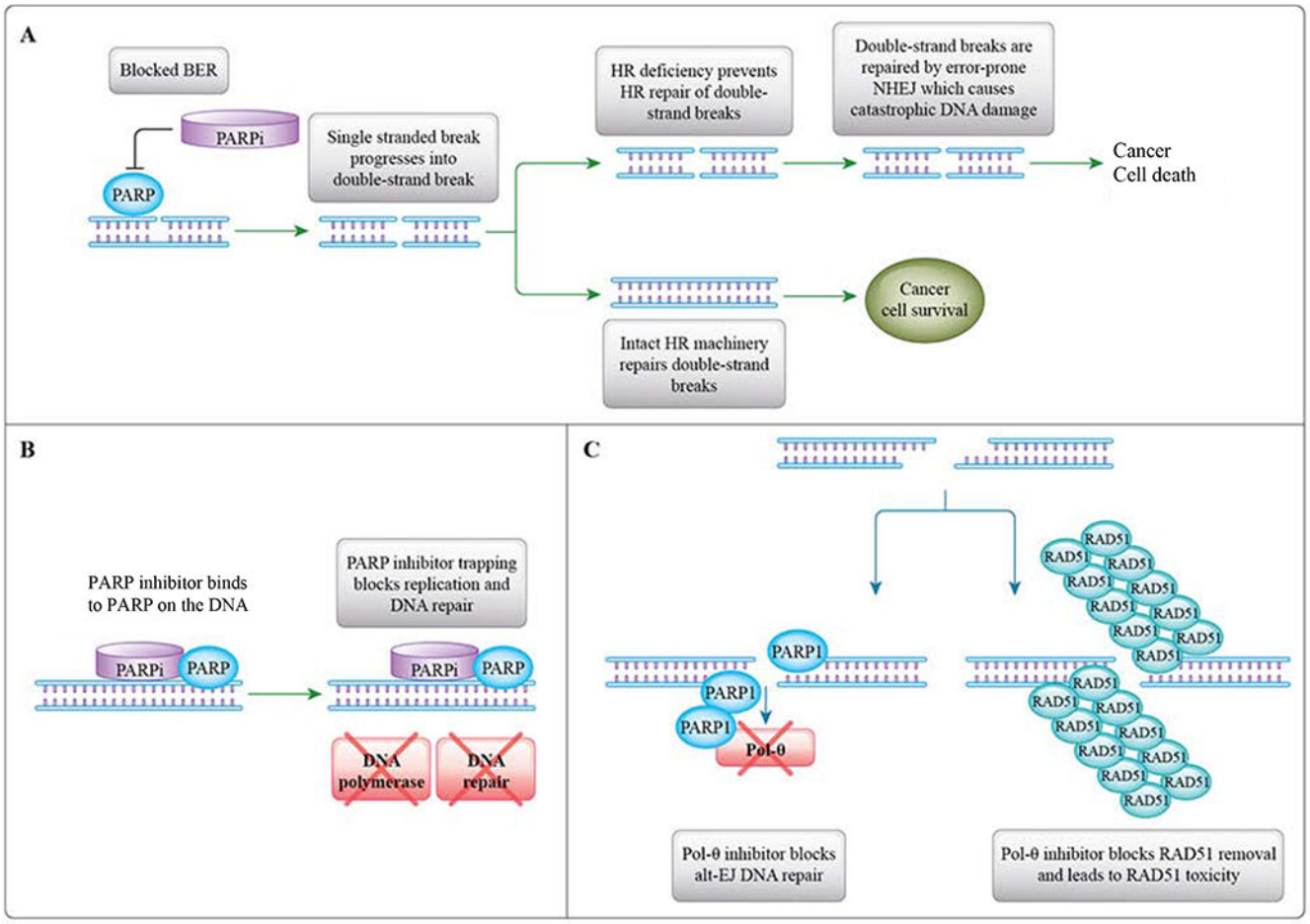

Figure 1: Mechanisms of PARP Inhibitor and POLΘ Inhibitor Cytotoxicity.

A. PARP inhibition blocks BER single-strand DNA repair. The inability to repair single-strand DNA breaks leads to the accumulation of double-strand DNA breaks. In HR-deficient cancers, cells are dependent on error-prone cNHEJ to repair double strand DNA breaks. The large number of genomic errors introduced by cNHEJ leads to catastrophic DNA damage. **B.** PARP inhibitor trapping blocks DNA replication and DNA repair. **C.** PolΘ inhibition blocks the Alt-EJ pathway from repairing double-stand DNA breaks. PARP and POLθ are known to cooperate in Alt-EJ. Inhibition of POLθ, and to a lesser extent, inhibition of PARP, results in the loss of Alt-EJ and the accumulation of toxic RAD51 foci. This mechanism provides a rationale for combining a PARP inhibitor with a POLθ inhibitor for cancer treatment. The combination may be synergistic since it leads to toxic RAD51 accumulation and cell death.

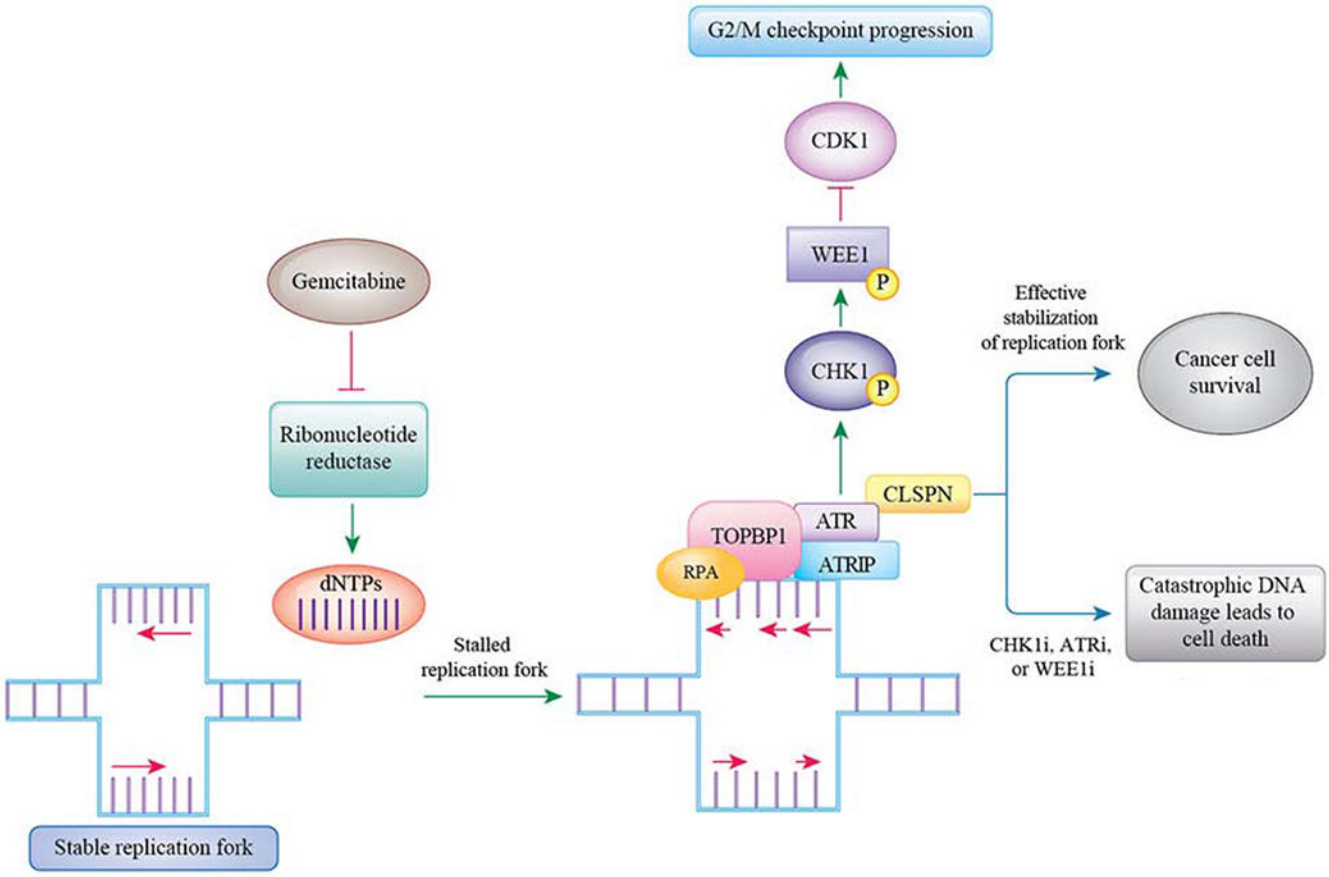

Figure 2: The ATR/CHK1/WEE1 pathway compensates for the replicative stress induced by gemcitabine.

Gemcitabine causes replicative stress by irreversibly inhibiting ribonucleotide reductase and thereby decreasing dNTP concentration. Decreased dNTP concentration causes stalled replication forks. In response to this replicative stress, the ATR/CHK1/WEE1 pathway is activated and this leads to stabilization of the replicative forks. However, inhibitors of the ATR/CHK1/WEE1 pathway block this compensatory pathway. This leads to persistence of unstable replication forks and ultimately causes genomic catastrophe leading to cancer cell death.

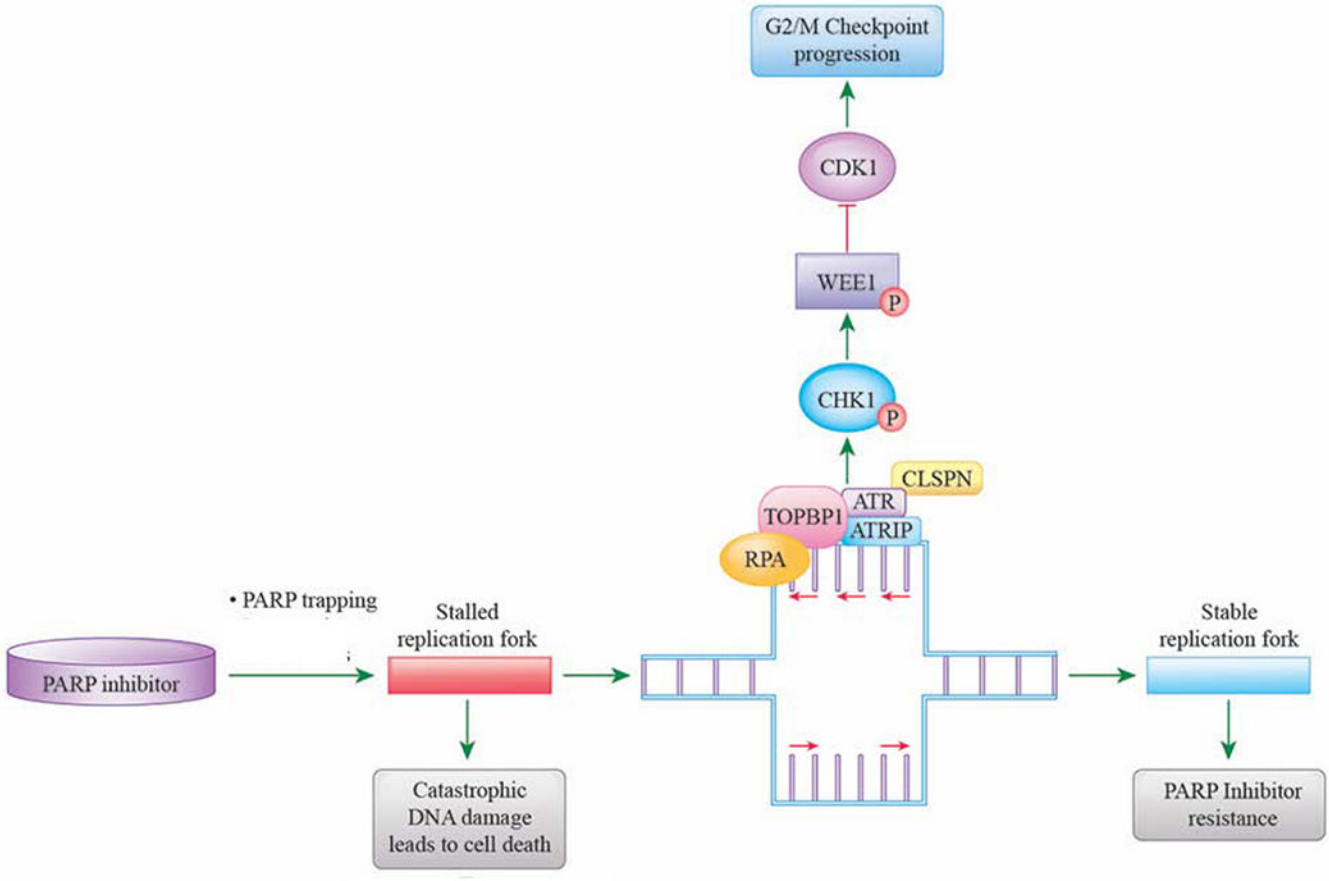

Figure 3: Hyperactivation of the ATR/CHK1/WEE1 pathway leads to acquired resistance to PARP inhibitors.

PARP inhibitors, through inhibition of single-strand DNA repair and PARP inhibitor trapping, causes stalled replication forks and ultimately cell death in HR-deficient cancers. Cancer cells can develop resistance to PARP inhibitors by hyperactivation of the compensatory ATR/CHK1/WEE1 pathway, leading to stabilization of replication forks.

Table 1.

DDR Inhibitors in Past or Present Clinical Evaluation

PARP Inhibitors	**CHK1 Inhibitor**	**ATR Inhibitors**
Niraparib (MK-4827)[1]	GDC-0575	Ceralasertib (AZD6738)
Olaparib (AZD-2281)[1]	LY3300054	BAY1895344
Talazoparib (BMN-673)[1]	MK-8776 (SCH-900776)	Berzosertib (M6620, VX-970)
Rucaparib (AG-014699)[1]	Prexasertib (LY2606368)	M4344 (VX-803)
Pamiparib (BGB-290)	SRA-737 (CCT245737)	
Veliparib (ABT-888)	AZD7762[2]	
CEP-9722[2]		
E7016 (GPI-21016)[2]		
INO-1001[2]		
DNA-PK Inhibitors	**WEE1 Inhibitors**	**ATM Inhibitors**
AZD7648	Adavosertib (AZD1775)	AZD0156
CC-115		M3541
M9831 (VX-984)		
Nedisertib (M3814)		

[1]FDA approved

[2]No longer in clinical trials

Table 2:

Predictive and Pharmacodynamic Biomarkers of DNA Repair Inhibitors

Drug	Target	Predictive Marker	Pharmacodynamic Marker[1]
PARPi	HR deficiency	HR gene mutation	↑γH2AX
		HRD Score (LOH), Signature 3	↓PARylation
		Unstable replication fork[2]	↑Rad51 (in HR proficient cells)[3]
		↑Polθ	
		Platinum sensitivity	
		Absent RAD51 foci	
		↓Monoubiquitination of FANCD2	
DNA-PKi	cNHEJ	Loss of Polθ or FEN1	
ATRi	RS[4]	ATM deficiency	↓pCHK1
		RS promoting genomic changes[4]	↑γH2AX
		Unstable replication fork	↑Unstable replication fork
		↑pRPA, ↑pKAP1	↑pKAP1
		RNase H2deficiency	
CHK1i	RS	p53 deficiency	↑γH2AX
		RS promoting genomic changes[4]	↑pCHK1
		Unstable replication fork	↑Unstable replication fork
		↑pRPA, ↑pKAP1	↑pRPA, ↑pKAP1
WEE1i	RS	p53 deficiency	↑pCHK1
		RS promoting genomic changes[4]	↑γH2AX
		Unstable replication fork	↑Unstable replication fork
		↑pRPA, ↑pKAP1	↑pRPA
			↓pCDK1 (pCDC2)
Polθi	Alt-EJ	HR deficieny[5]	↑Rad51
		↑Polθ	↑γH2AX
		↓cNHEJ	↑Rad51 (in HR proficient cells)

[1] Pharmacodynamic markers are a metric of target and/or pathway engagement

[2] Unstable replications forks are evaluated in the laboratory with DNA fiber assays

[3] RAD51 foci increase in HR proficient cells. RAD51 foci are absent in HR deficient cells

[4] Replicative stress (RS) promoting genomic changes include *CCNE1* and *MYC* amplification and *FBXW7* mutations

[5] Polθ shares the same predictive biomarkers as PARP inhibitors.

Table 3.

Mechanisms of PARP Inhibitor Resistance

Mutation	Mechanism	References
Acquired *BRCA*1/2 Gene Mutation	Somatic Reversion	(Sakai et al., 2008)
Decrease in: **53BP1** **RIF1** **REV7** **SHLD1, 2, 3**	Inactivation of SHLD complex and restoration of HR	(Dev et al., 2018; Jaspers et al., 2013; Nacson et al., 2018; Tomida et al., 2018; Xu et al., 2015)
Decrease in: **PTIP** **EZH2**	Prevention of fork degradation	(Ray Chaudhuri et al., 2016; Rondinelli et al., 2017a)
Amplification TRIP13	Removal of SHLD complex and restores HR	(Clairmont et al., 2020)
PARP1 loss	Loss of target	(Pettitt et al., 2018)
Increased P-glycoprotein	Increased drug efflux	(Henneman et al., 2015)
Decreased SLFN11	Fork stabilization	(Lok et al., 2017)
Decreased PARG	Increased PAR chains	(Gogola et al., 2018)
Increased TIRR	Blocks 53BP1 binding	(Drane et al., 2017)
Decreased DNLL1	Increased DSB end resection	(He et al., 2018)

Table 4.

Selected Clinical Trials Combinations Involving ATM, ATR, CHK1, DNA-PK, and WEE1 Inhibitors

Combination	Trial Number	Phase	Tumor Type
ATMi/Chemotherapy			
AZD0156/FOLFIRI	NCT02588105	1	Solid Tumor
ATMi/PARPi			
AZD0156/Olaparib	NCT02588105	1	Solid Tumor
ATRi/PARPi			
Olaparib/Ceralasertib	NCT03462342	2	Ovarian Cancer
Niraparib/M4344	NCT04149145	1	Ovarian Cancer
ATRi/Chemotherapy			
Ceralasertib/Gemcitabine	NCT03669601	1	Solid Tumors, Ovarian Cancer
ATRi/PD1i			
BAY1895344/Pembrolizumab	NCT04095273	1	Solid Tumor
CHK1i/PARPi			
Prexasertib/Olaparib	NCT03057145	1	Solid Tumor
CHK1i/Chemotherapy			
LY2880070/Gemcitabine	NCT02632448	1	Solid Tumor
CHK1i/PD1i			
Prexasertib/LY3300054	NCT03495323	1	Solid Tumor
DNA-PKi/PD1i			
M3814/Avelumab	NCT03724890	1	Solid Tumor
DNA-PKi/Radiation+/−Chemotherapy			
M3814/Capecitabine/Radiation	NCT03770689	1	Solid Tumor
WEE1i/PARPi			
Olaparib/AZD1775	NCT04197713	1	Solid Tumors
WEE1i/Chemotherapy			
AZD1775/Cisplatin	NCT03012477	2	Breast Cancer
WEE1i/PD1i			
AZD1775/Durvalumab	NCT02617277	1	Solid Tumors