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Restored replication fork stabilization, a mechanism of PARP inhibitor resistance, can be overcome by cell cycle checkpoint inhibition

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ABSTRACT

Poly(ADP-ribose) polymerase (PARP) inhibition serves as a potent therapeutic option eliciting synthetic lethality in cancers harboring homologous recombination (HR) repair defects, such as *BRCA* mutations. However, the development of resistance to PARP inhibitors (PARPis) poses a clinical challenge. Restoration of HR competency is one of the many molecular factors contributing to PARPi resistance. Combination therapy with cell cycle checkpoint (ATR, CHK1, and WEE1) inhibitors is being investigated clinically in many cancers, particularly in ovarian cancer, to enhance the efficacy and circumvent resistance to PARPis. Ideally, inhibition of ATR, CHK1 and WEE1 proteins will abrogate G2 arrest and subsequent DNA repair via restored HR in PARPi-treated cells. Replication fork stabilization has recently been identified as a potential compensatory PARPi resistance mechanism, found in the absence of restored HR. ATR, CHK1, and WEE1 each possess different roles in replication fork stabilization, providing different mechanisms to consider when developing combination therapies to avoid continued development of drug resistance. This review examines the impact of ATR, CHK1, and WEE1 on replication fork stabilization. We also address the therapeutic potential for combining PARPis with cell cycle inhibitors and the possible consequence of combination therapies which do not adequately address both restored HR and replication fork stabilization as PARPi resistance mechanisms.

Introduction

Poly(ADP-ribose) polymerase (PARP) inhibition is at the forefront of cancer treatment, particularly in cancers with homologous recombination (HR) repair defects such as *BRCA* mutations [1,2]. PARP1 is the most abundant PARP family member and is involved in multiple DNA damage repair pathways, including base excision repair (BER), HR repair, and non-homologous end joining (NHEJ) [3,4]. Upon sensing DNA damage, PARP1 undergoes a conformational change to increase its catalytic activity for adding poly(ADP-ribose) chains (PARylation) to various DNA repair enzymes, histones and itself [5,6]. PARP2 is less abundant and contributes 5% to 10% of the total PARP activity [7,8]. AutoPARylation of PARP1 and PARP2, and PARylation of chromatin proteins promotes recruitment of repair factors and releases PARP1 and PARP2 from DNA to allow repair [5,9]. All clinically active PARP inhibitors (PARPis) are designed to compete with NAD+, a substrate of poly(ADP-ribose) chain, and inhibit the enzymatic activity of PARP1 and PARP2 [10].

Defects in HR repair offer a therapeutic opportunity in which DNA repair inhibitors, e.g. PARPis, can be used to induce lethal DNA double stranded breaks (DSBs). PARPis induce DSBs via catalytic inhibition [1,2] and PARP-DNA trapping [11–13], by which PARPis prompt synthetic lethality in BRCA deficient cells. This synthetic lethality due to BRCA loss and PARPi has been extensively investigated in the preclinical and clinical settings, particularly in *BRCA* mutated ovarian cancer [14–18].

Ovarian cancer is the most lethal gynecologic cancer among women world wide accounting for an estimated 152,000 deaths annually [19,20]. Molecular profiling has identified that nearly 40% of high grade serous ovarian cancer (HGSOC) have mutations in HR genes [21–23]. Results from clinical trials investigating the benefit of PARPis in ovarian cancer led to the United States Food and Drug Administration approving three PARPis, olaparib, rucaparib and niraparib. Olaparib and rucaparib are approved for the treatment of germline *BRCA* and both germline and somatic *BRCA* mutated advanced ovarian cancer patients, respectively, who have previously been treated with chemotherapy [15,24]. Also, all three PARPis are licensed for use in maintenance treatment of recurrent ovarian cancer with complete or partial response to platinum-based therapy [25–28]. Two additional PARPis, talazoparib and veliparib, are in advanced clinical trials. PARPi

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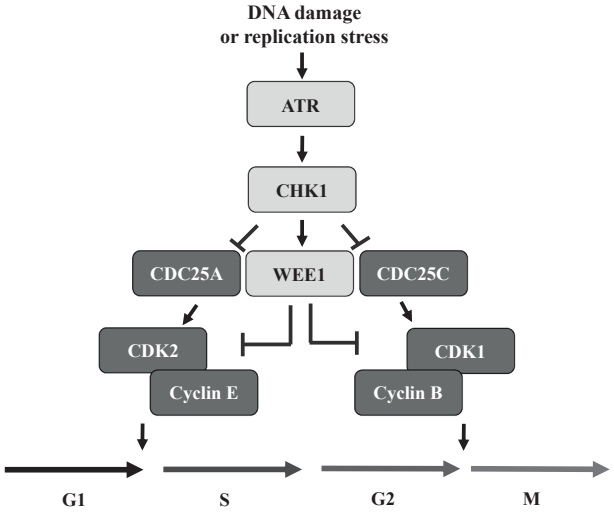

Fig. 1. G2/M checkpoint signaling pathway. ATR (ataxia telangiectasia and Rad3-related) is activated by single stranded DNA damages or replication stress and phosphorylates CHK1. Upon phosphorylation, CHK1 is activated and subsequently activates WEE1 via phosphorylation. Simultaneously, CHK1 phosphorylates and inactivates the CDC25A and CDC25C phosphatases. Activated WEE1 then phosphorylates CDK1 and CDK2 to promote G1/S and G2/M cell cycle arrest.

treatment however primarily results in partial tumor regression with rare complete responses and most overall responses are short lived (< 1 year) with the emergence of resistance [29]. Work is now ongoing to optimize PARPi combination approaches to broaden the target patient population and to avoid development of resistance. Combination with cell cycle checkpoint inhibitors (hereafter described as cell cycle inhibitors) is becoming a testable therapeutic option to enhance the anti-tumor activity of PARPis.

Cells initiate a multitude of responses to protect the genome and ensure survival in response to DNA damage [30]. These responses include activation of cell cycle checkpoints, subsequent cell cycle arrest to provide the cell time to repair damaged DNA, and activation of the appropriate DNA repair mechanisms to efficiently complete repair. DSBs induced by PARPis are generated during S phase through collision of replication forks with unrepaired SSBs and PARP-DNA trapping lesions and would normally result in halting of the S phase checkpoint [13]. However, ovarian cancer, like many others, have mutant or null p53 causing dysfunction of the p53-dependent S phase checkpoint [22]. These cancers instead rely heavily on G2 checkpoint stoppage to facilitate DNA damage repair (Fig. 1) [31]. ATR (ataxia telangiectasia and Rad3-related) is a central checkpoint protein kinase that is activated by single strand DNA (ssDNA) damage, including the resected ends of DNA DSBs and stalled replication forks. ATR activation induces a global shutdown of origin firing and slows down fork speed through activation of checkpoint kinase 1 (CHK1; a critical component of G2 checkpoint arrest) and inactivation of cyclin-dependent (CDK), specifically CDK1 and CDK2 (CDK1/2) [32,33]. WEE1 kinase, similarly integral for the G2 checkpoint, also keeps CDK1/2 inactive by phosphorylating CDK1/2 directly [34]. Therefore, the combination of cell cycle (ATR, CHK1, and WEE1) inhibitors with PARPis limits the time given to repair DNA, by restored HR, and promotes replication of damaged DNA resulting in cell death. This indication has spurred several clinical trials combining PARPis and cell cycle inhibitors (Table 1).

Recent studies implicated replication fork stabilization (hereafter described as fork protection) as a compensatory PARPi resistance mechanism in the absence of HR competency [35–37]. Clinically, fork protection may pose a challenge as this process can continue to promote the development of PARPi resistance even though HR is abrogated by ATR, CHK1, and WEE1 inhibitors. Therefore, it is essential to target both restored HR and fork protection to fully combat PARPi resistance with combination therapy. In this review, we address the differential roles of ATR, CHK1, and WEE1 in fork protection and the therapeutic benefit of combining PARPis with cell cycle inhibitors. Additionally, we discuss the potential for the continued development of PARPi resistance if both restored HR and fork protection are not addressed.

Cooperative fork destabilization and synthetic lethality

Stabilization of stalled DNA replication forks, which includes the process of replication fork reversal, protection and restart, is gaining attention in the field of DNA damage response (DDR; Fig. 2) [38,39]. Following replication perturbation, replication forks remodel into four-way junctions, known as fork reversal, to allow more time for repair of DNA lesions, avoid conversion of single stranded breaks (SSBs) into DSBs, and enable excision repair by re-positioning a lesion in double-stranded DNA [40]. The reversed forks should be protected from extended nucleolytic degradation, which is an essential step to ensure genome stability.

PARP1 is implicated in fork protection and cooperates with BRCA2 in this process [41]. BRCA2 and PARP1 independently protect stalled replication forks from MRE11-dependent degradation; loss of both BRCA2 and PARP1 results in heightened MRE11-mediated degradation [42]. Additionally, PARP1 and BRCA2 stabilize RAD51 at stalled replication forks in a HR-independent manner and RAD51 is involved in restoration of fork protection [43,44]. Together these data describe a collaborative role for PARP1 and BRCA2 in protecting stalled replication forks by stabilizing RAD51 at stalled forks and preventing MRE11-mediated degradation. Moreover, RECQ1 is a human helicase specialized in restarting reversed forks following replication stalling [43]. PARP1-mediated PARylation of RECQ1 counteracts untimely RECQ1-dependent replication fork restart, thus preventing DSB formation [43]. PARP1 is therefore required to accumulate reversed forks while its activity is dispensable for their formation. In this context, PARPis would promote toxic fork restart generating DSBs, which can explain another potential mechanism of synthetic lethality in BRCA deficient cancers.

The situation is possibly more complex as recent studies show that PARP1 inhibition protects stalled replication forks from MRE11-mediated degradation and rescues the viability of cells undergoing *BRCA2* null crisis, defined by excessive fork degradation and collapse [45,46]. Ding et al. proposed that this rescue of BRCA2 deficient cells by PARP inhibition may be due to transiently protected replication forks and that this transient fork protection may be sufficient for the initial survival of BRCA2 deficient cells. Since PARPis are applied for maintenance therapy, this unfavorable possibility should be considered in incoming clinical assessment. Nevertheless, the acquired *BRCA2* null cells will most likely be killed by the inability to repair these lesions.

Further complexity comes with another recent report by Feng et al. [47]. In their setting using BRCA2 conditional non-transformed human mammary epithelial MCF10A cells, neither PARP1 deletion/inhibition or MRE11 deletion conferred a growth advantage in *BRCA2* deficient cells even though these deletions restored fork protection. Furthermore, PARP1 depletion failed to suppress cisplatin induced γ H2AX formation in *BRCA2* deficient cells. Though authors conclude that HR, and not fork protection, is primarily responsible for suppressing replication stress and supporting cell viability, it is important to note they exclusively examine non-transformed cells. Authors also noted that in cells with defective p53 and HR, fork protection may play a role in cell survival, suggesting a potential compensatory role for this resistance mechanism.

Restored fork protection as a mechanism PARPi resistance

Stabilization of stalled DNA replication forks is recently identified as mechanism of PARPi resistance [37,44,48,49]. Reversed forks are extensively degraded in cells with defective BRCA2, RAD51, FANCD2 or

Table 1
Ongoing PARPi and cell cycle inhibitor combination clinical trials.

NCT number	Therapeutic intervention	Disease	Trial status	Phase
03057145	Olaparib + Prexasertib (CHK1 inhibitor)	Advanced solid tumor	Recruiting	I
02511795	Olaparib + AZD1775 (WEE1 inhibitor)	Refractory solid tumor	Recruiting	I
02723864	Veliparib + VX-970 (ATR inhibitor) + Cisplatin	Refractory solid tumors	Recruiting	I
02264678	AZD6738 (ATR inhibitor) alone or + olaparib or + carboplatin or + MEDI4736	Advanced solid malignancy, head and neck squamous cell carcinoma, ATM proficient/deficient non-SCLC, gastric and breast cancer	Recruiting	I/II
03330847	Olaparib alone or + AZD1775 or + AZD6738	Metastatic triple negative breast cancer	Recruiting	II
02576444	Olaparib alone or + AZD1775 or + AZD6738 or + AZD5363 (AKT inhibitor)	Tumors harboring either *TP53* or *KRAS* mutations	Recruiting	II
03462342	Olaparib + AZD6738 (CAPRI: combination ATR and PARP Inhibitor)	Recurrent ovarian cancer	Recruiting	II
03428607	Olaparib + AZD6738	Relapsed small cell lung cancer (SCLC) patients	Not yet recruiting	II
03579316	Adavosertib (AZD1775) +/− olaparib	Recurrent ovarian cancer patients who have progressed on prior PARP inhibition	Not yet recruiting	II

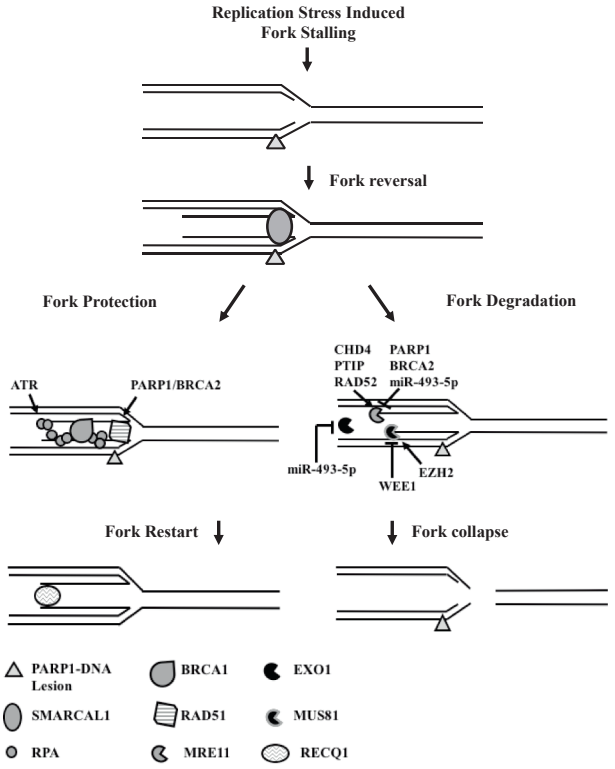

Fig. 2. Consequence of replication fork stalling. In response to replication stress, replication forks stall at the site of damage (△). Stalled forks can then be reversed by SMARCAL1 and these reversed forks can undergo fork protection by replication protein A (RPA; recruited by ATR), BRCA2, and/or RAD51 (recruited by PARP1 and BRCA2) and be restarted by RECQ1. Alternatively, these reversed forks can be degraded by EXO1, MRE11 (recruited by PTIP, CHD4, RAD52), and/or MUS81 (recruited by EZH2) mediated fork degradation and subsequent fork collapse and cell death. Notably, PARP1 and BRCA2 inhibits MRE11 mediated fork degradation and miR-493-5p blocks both MRE11 and EXO1 activity, supporting the role of PARP1, BRCA2, and miR-493-5p in fork protection and PARP inhibitor resistance.

FANCA, through uncontrolled resection by the MRE11 and EXO1 nucleases [50–53]. Defects in BRCA2, RAD51, FANCD2, and FANCA are also known to sensitize cancer cells to PARPis [15,54,55]. Fork degradation by MRE11 in *BRCA1* and/or *BRCA2* deficient cells is promoted by the MLL3/4 complex protein PTIP, the nucleosome remodeling factor CHD4, and RAD52 (Fig. 2) [37,53]. Inactivation of these protective factors against MRE11, and inhibition of MRE11, reduced accumulation of chromosomal aberrations in response to olaparib and/or cisplatin in *BRCA* deficient cells, suggesting loss of these proteins protected cells from the toxic effects of excess fork degradation [37,53]. Loss of the *EZH2/MUS81* axis, known for its endonuclease activity, also promoted resistance to rucaparib and cisplatin in *BRCA2*-deficient ovarian cancer cells because of heightened fork protection [48]. Notably, loss of *EZH2* did not impact MRE11-mediated fork degradation, indicating that the *EZH2/MUS81* axis function independently of MRE11 [48]. Genome wide RNA sequencing of tumors from primary and relapsed ovarian cancer patients identified overexpression of miR-493-5p to be associated with platinum and PARPis resistance in *BRCA2* mutated patients [49]. Overexpression of miR-493-5p prevented fork degradation by downregulating *MRE11* and *EXO1* [49]. These data consolidate that restoration of fork protection contributes to acquired PARPi resistance in *BRCA* deficient setting and may have clinical implications.

Mechanisms of resistance to PARPis by HR restoration

Whereas fork protection is a burgeoning mechanism of PARPi resistance, restoration of HR is a well-known PARPi resistance mechanism [56,57]. HR function is restored by secondary reversion mutations of *BRCA1, BRCA2* and *RAD51* isoforms [56,57]. Clinically, secondary mutations restoring *BRCA* function were found in patients with germline *BRCA* mutation associated ovarian and breast cancer upon acquired resistance to PARPis and/or platinums [58,59]. Additionally, four *RAD51C* secondary mutations and one *RAD51D* secondary mutation were identified upon development of resistance in testing paired biopsies from 12 patients with platinum sensitive recurrent HGSOC who received rucaparib on the ARIEL2 Part 1 trial [57].

Restored HR is newly attributed to the shieldin complex [60–63]. Shieldin was identified as a four-subunit ssDNA-binding complex consisting of REV7, c20orf196 (SHLD1), FAM35a (SHLD2), and FLJ26957 (SHLD3) [60]. Shieldin was shown to accumulate at DSB sites and bind to ssDNA to prevent DSB resection and facilitate NHEJ [60,61]. Therefore, loss of shieldin impairs NHEJ and promotes resistance to PARP inhibition in *BRCA1* knock out cells due to restored HR [61,63]. The recent discovery of shieldin demonstrates the complexities involved in HR restoration during the development of PARPi resistance and reveals that there is more to be uncovered regarding this resistance

mechanism.

Many of the genes involved in HR, such as *BRCA1, BRCA2, RAD51* and *MRE11*, are also involved in fork protection; however, mechanisms of resistance occurring through restoration of HR and fork protection are mutually exclusive [35,37,48,49]. Inactivation of *MUS81* or loss of *PTIP* in *BRCA* mutant cells restores fork protection, but has no impact on HR. Moreover, overexpression of miR-493-5p also did not restore HR [49]. Notably, restoration of HR, as measured by RAD51 focus formation, is shown to be acquired prior to restoration of fork protection in a panel of isogenic olaparib-resistant *BRCA1* mutant ovarian cancer cells [35]. This implies that restoration of HR and fork protection are independent, potentially clinically relevant, mechanisms of PARPi resistance. Hence, both restored HR and fork protection that should be considered when devising PARPis therapies as single agents as well as in combination to sustain therapeutic benefit.

Therapeutic potential of cell cycle inhibitors in combination with PARPis

Combinations of PARPis with different ATP-competive cell cycle inhibitors are being investigated in clinical trials (Table 1). The rationale of these combinations is to limit the time the cell has to repair damaged DNA and to dysregulate replication, resulting in cell death.

Recent data indicate that ATR may function independently of CHK1 to protect replication forks [64,65]. When cells were analyzed for replication induced DSBs in response to UV irradiation, only ATR inhibition with caffeine enhanced DSB formation, while inhibition of CHK1 with CEP3891, Go6976, or SB218078 did not [64]. Further, ATR inhibition resulted in hyperactive SMARCAL1, a translocase promoting fork reversal, leading to increased fork reversal and subsequent fork degradation, which was CHK1-independent [44,65]. Activated ATR also protected ssDNA tracts by supplying replication protein A (RPA) and preventing stalled forks from collapsing (Fig. 2) [44,66]. Synthetic lethality was demonstrated in MCF-7 human breast cancer cells treated with ATR (VE821) and CHK1(AZD7762) inhibitors [66]. Sanjiv et al. showed that CHK1 inhibition activated ATR by increasing the overall CDK2-mediated replication stress and the amount of ssDNA tracts [66]. Activated ATR then protected ssDNA tracts by supplying RPA [44,66]. Hence, the addition of ATR inhibitors resulted in a high number of stalled replication forks collapsing into toxic, irreparable DSBs, killing the cancer cells [66,67].

The differences observed between ATR and CHK1 in fork protection extend to their therapeutic utility in combination with PARPis. For instance, the ATR inhibitor VE821 and ATR depletion by siRNA sensitized *BRCA* wild type, p53 mutant OVCAR8 ovarian cancer cells to a broad range of chemotherapeutic agents, including gemcitabine and the PARPi, veliparib [68]. Conversely, CHK1 inhibition with MK8776 only sensitized OVCAR8 cells to gemcitabine and not veliparib [68]. Similarly, the ATR inhibitor AZD6738 was found to sensitize PEO1 (*BRCA2* mutant), PEO4 (*BRCA2* reversion mutation), and JHOS4 (*BRCA1* wild type) ovarian cancer cells to olaparib more effectively than the CHK1 inhibitor MK8776 [69]. AZD6738 and olaparib also induced increased replication fork collapse and chromosomal gaps and breaks compared to the CHK1 inhibitor MK8776 + olaparib, even though both AZD6738 and MK8776 abrogated PARPi induced G2/M arrest [69]. Differential effects between ATR and CHK1 inhibition in combination with PARPi is also evident in *in vivo*. AZD6738 in combination with olaparib resulted in tumor regression and eradication in *BRCA2* mutant orthotopic patient derived xenograft models, whereas treatment with MK8776 and olaparib caused only tumor suppression in the same xenograft models [69]. Further, VE821 resensitized olaparib-resistant *BRCA1* mutant UWB1.289 ovarian cancer cells to olaparib more effectively than MK8776 at comparable micromolar doses [35]. These results support the possible separation of function between ATR and CHK1 and implicate that this separation of function with ATR and CHK1 may be clinically relevant in PARPi resistant settings.

WEE1 is also implicated in fork protection. Dominguez-Kelly et al. showed loss of WEE1 resulted in MUS81-mediated DNA cleavage [70,71]. Additionally, co-immunoprecipitation revealed WEE1 and MUS81 interact directly in p53 wild type osteosarcoma U2OS cells [70]. As downregulation of MUS81 is associated with fork protection [48,52], this implicates a role for WEE1 in protecting replication forks by negatively regulating MUS81-mediated degradation (Fig. 2) [70]. WEE1 inhibition with AZD1775 was found to synergize with the CHK1 inhibitor MK8776 in a panel of 39 cell lines, including the *BRCA1* wild type ovarian cancer cell line A2780 [72]. Synergistic cytotoxicity effects were also apparent in colorectal cancer and ovarian carcinoma xenografts [72]. This synergy suggests that WEE1 and CHK1 have complementary, though differing, roles in response to chemotherapy that may involve WEE1-mediated fork protection.

WEE1 inhibition may be more cytotoxic than ATR and CHK1 inhibition when used in combination with cisplatin and gemcitabine in triple negative breast cancer, pancreatic cancer, and osteosarcoma cells [73,74]. WEE1 inhibition reversed cisplatin induced replication fork stalling, as measured by EdU incorporation, more effectively than both ATR and CHK1 inhibition in *BRCA1* wild type triple negative breast cancer cells [73]. WEE1 activity is required to sustain ATR and CHK1 activity, as inhibition of WEE1 abrogates ATR and CHK1 phosphorylation in response to gemcitabine in both pancreatic adenocarcinoma Panc1 and osteosarcoma U2OS cells [74]. These data suggest that WEE1 may have a more robust role in DNA replication compared to ATR and CHK1 due to a requirement for WEE1 in the maintaining ATR and CHK1 activatin. However, this assumption would need to be verified in further studies.

Together these data imply that the differences observed in the antitumor activity of these proteins upon pharmacological inhibition in combination with PARPis may be due to the possible unique cellular functions of ATR, CHK1 and WEE1 regarding fork protection. Currently, clinical trials (Table 1) focus on determining the impact of ATR, CHK1, or WEE1 inhibition in combination with PARPi. The combination ATR and PARP inhibitor (CAPRI) trial using AZD6738 and olaparib recently opened for recurrent ovarian cancer (NCT03462342). The CHK1 inhibitor LY2606368 (prexasertib) and olaparib combination is also being investigated in patients with advanced solid tumors (NCT03057145). A randomized phase 2 study beginning in September 2018 will utilize the WEE1 inhibitor AZD1775 in combination with olaparib for recurrent ovarian cancer patients who have progressed on PARPis (NCT03579316). These studies include patients who were previously treated with PARPis and will shed the light on the potential improvement of drug response with checkpoint inhibition in PARPi resistant patients. Furthermore, all three trials require participants have adequate bone marrow function as bone marrow suppression, in addition to gastrointestinal toxicity, is a major side effect of cell cycle inhibition [31]. Results from these trials, and others like them (Table 1), will provide the scientific community with much needed data regarding the ability of ATR, CHK1, and WEE1 inhibitors to overcome PARPi resistance.

The rational for current clinical trials focuses on the roles of ATR, CHK1, and WEE1 in inducing G2 arrest with the goal of eliminating this arrest and preventing DNA repair, via HR restoration, in PARPi treated tumors. However, if fork protection should play a compensatory role in these tumors, then resistance may still develop through restoration of this process given ATR, CHK1 and WEE1 potentially function differently in regard to fork protection. This also could create a possible clinical barrier to combination therapies as some combinations may only impact one facet of PARPi resistance and thus permit further development of PARPi resistance (Fig. 3). Therefore, it is critical use a cell cycle inhibitor that affects both HR and fork protection when designing combination trials utilizing PARPis and cell cycle inhibitors. Additionally, careful examination of potential pharmacodynamic markers for replication fork protection, e.g., RPA, in pre-treatment and at progression tissue samples and independent validation will be necessary to

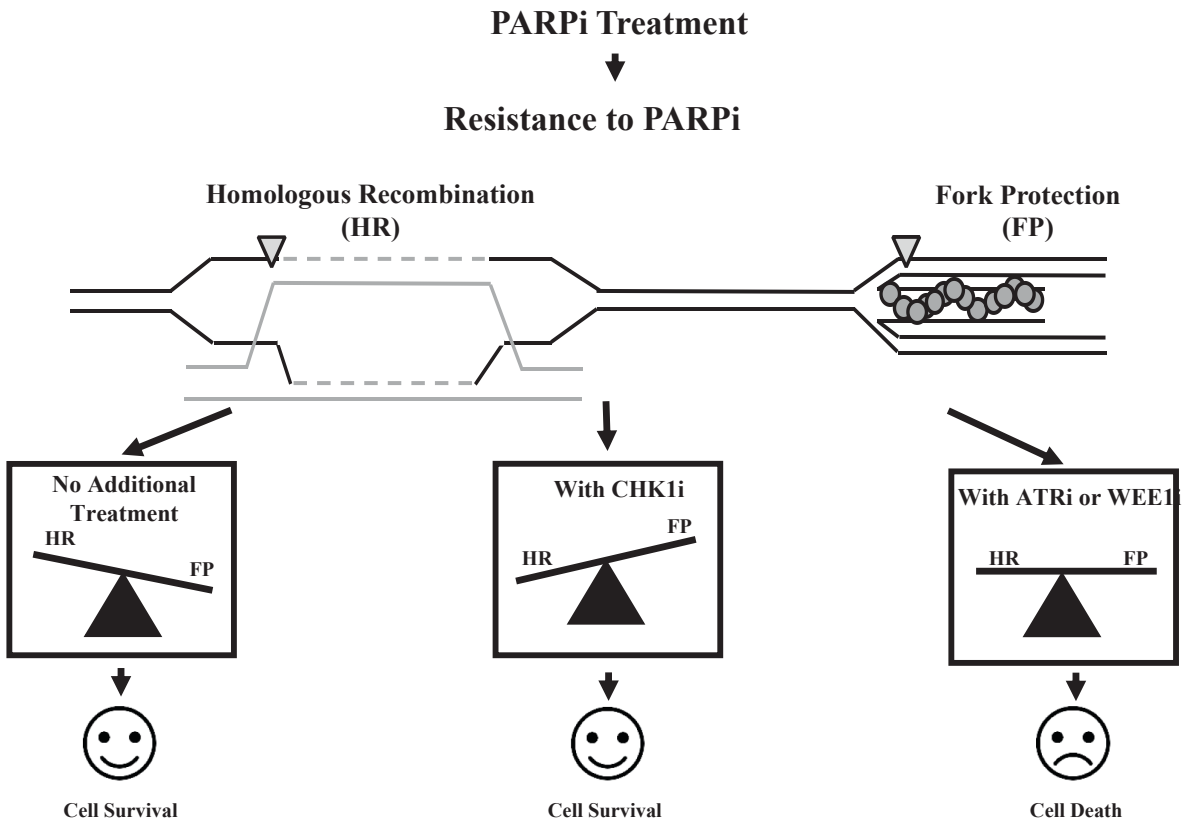

Fig. 3. Development of PARP inhibitor resistance. PARP inhibitor (PARPi) resistance develops mainly through restoration of homologous recombination (HR) with fork protection (FP) potentially playing a compensatory role in this process (left box). Combination therapies with cell cycle inhibitors that only disrupt HR, such as CHK1 inhibitors (CHK1i), may result in continued resistance mediated by fork protection (middle box). In contrast, combination therapies utilizing cell cycle inhibitors that target both restoration of HR and FP (e.g. ATR inhibitors; ATRi or WEE1 inhibitors; WEE1i) may prevent PARPi resistance and enhance cell death (right box). ▽:PARP-DNA lesion ●:RPA.

improve therapeutic potential of these drugs.

Conclusion

The use of PARPis are gradually evolving, including combination strategies with cell cycle checkpoint inhibitors in ovarian cancers with and without *BRCA* mutations. An improved understanding of the mechanisms underlying PARPis and cell cycle inhibitors clinical resistance will be important to enable the development of new approaches to increase efficacy. In particular, developing biomarkers to identify tumors with heightened HR and fork protection ability may classify a subgroup of patients who may gain the most benefit from combined PARP and cell cycle inhibitors. It would be important to design and interpret clinical trials based on biological hypotheses and robust preclinical data. This is a field rich in opportunity, and the coming years should see a better understanding of which cancer patients we should treat with PARPis and a cell cycle inhibitors and where these agents should come in over the course of treatment.

Conflict of interest

The authors declare no potential conflicts of interest.

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