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DNA replication stress and emerging prospects for PARG inhibitors in ovarian cancer therapy

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ABSTRACT

Poly (ADP-ribosyl)ation has central functions in maintaining genome stability, including facilitating DNA replication and repair. In cancer cells these processes are frequently disrupted, and thus interfering with poly (ADP-ribosyl)ation can exacerbate inherent genome instability and induce selective cytotoxicity. Indeed, inhibitors of poly (ADP-ribose) polymerase (PARP) are having a major clinical impact in treating women with *BRCA*-mutant ovarian cancer, based on a defect in homologous recombination. However, only around half of ovarian cancers harbour defects in homologous recombination, and most sensitive tumours eventually acquire PARP inhibitor resistance with treatment. Thus, there is a pressing need to develop alternative treatment strategies to target tumours with both inherent and acquired resistance to PARP inhibition. Several novel inhibitors of poly (ADP-ribose)glycohydrolase (PARG) have been described, with promising anti-cancer activity *in vitro* that is distinct from PARP inhibitors. Here we discuss, the role of poly (ADP-ribosyl)ation in genome stability, and the potential for PARG inhibitors as a complementary strategy to PARP inhibitors in the treatment of ovarian cancer.

1. Introduction

Inhibitors of poly (ADP-ribose) polymerase (PARP) 1 and 2 have revolutionised the treatment of *BRCA*-mutant tumours; for example, in *BRCA*-mutant high-grade serous ovarian carcinoma (HGSOC) treatment with PARP inhibitors significantly improves survival (Moore et al., 2018). *BRCA*-mutant tumours are sensitive to PARP inhibitors because they possess a defect in homologous recombination (HR). Up to 50% of HGSOC tumours are HR-deficient and are potentially amenable to treatment with these agents (TCGA, 2011; Yi et al., 2019). Nevertheless, this leaves a remaining subset of tumours that are intrinsically resistant to PARP inhibitors. Moreover, in patients that receive PARP inhibitors, almost 90% eventually develop resistance (Hodgson et al., 2018). Therefore, additional therapeutic strategies are required to treat patients with ovarian cancer who are unlikely to benefit from PARP inhibitors. In this review, we focus on one such alternative strategy, inhibitors of the enzyme poly (ADP-ribose) glycohydrolase (PARG). First, we will outline the opposing activities of PARP and PARG enzymes in poly (ADP-ribose) (PAR) turnover and downstream processes. This will be followed by reviewing the evidence for PARG inhibitors as an alternative therapeutic strategy, discussing the distinct yet complementary activity to that of PARP inhibitors.

2. Poly(ADP-ribosyl)ation enzymatic biology

2.1. The PARP family

ADP-ribosylation is a post-translational modification whereby ADP-ribose subunits are attached onto acceptor proteins (Fig. 1). The reaction is catalysed by ADP-ribosyltransferases (ARTs), using nicotinamide adenine dinucleotide (NAD+) as a substrate. Within this group of enzymes is the PARP family, of which at least 17 members have been identified, characterised by their conserved catalytic ADP-ribosyl transferase diptheria toxin-like domain (ARTD) (Hottiger et al., 2010). The majority of PARP enzymes catalyse the transfer of a single ADP-ribose unit. By contrast, five enzymes catalyse poly (ADP-ribosyl)ation (PARylation), namely,

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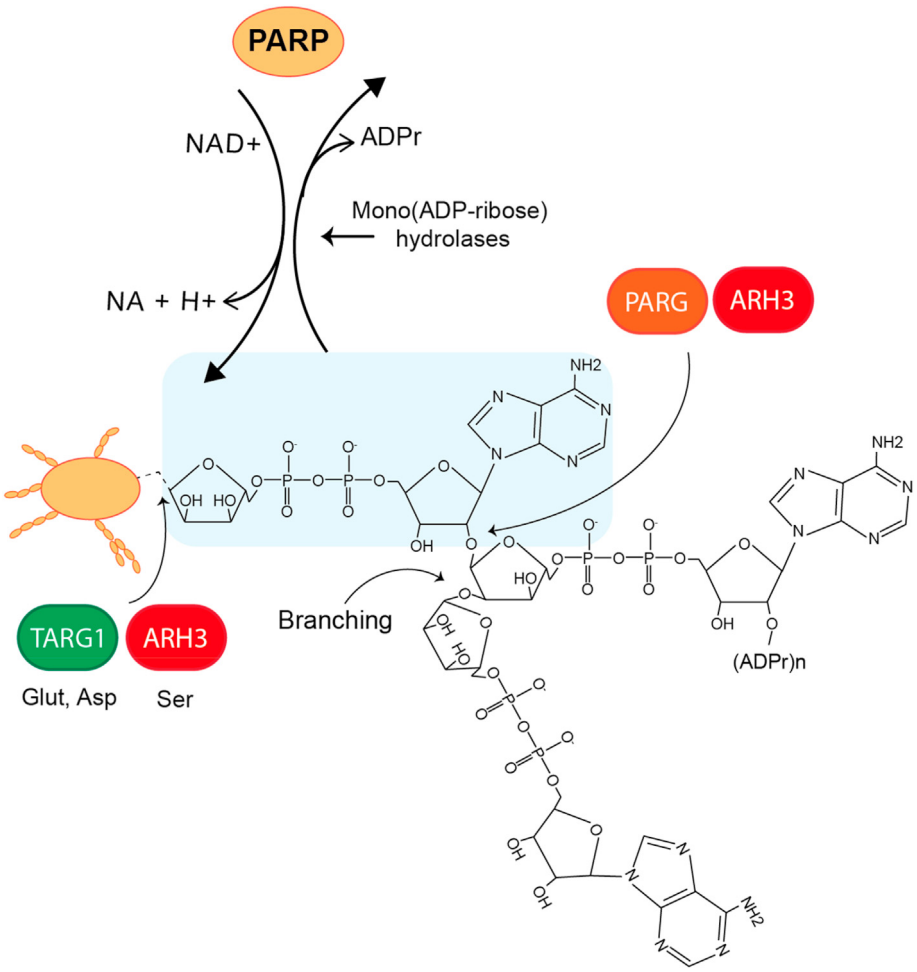


Fig. 1. The Poly (ADP-ribosyl)ation reaction.
Poly (ADP-ribosyl)ation is catalysed by PARP using NAD + as a substrate, and releasing nicotinamide (NA) and H+ as byproducts. The blue box indicates a single ADP-ribose (ADPr) subunit. Multiple subunits can be attached onto acceptor proteins into linear or branched poly (ADP-ribose) chains. PARG cleaves the O-Glycosidic bond between adjacent subunits releasing ADP-ribose. The dotted line attached to the acceptor protein indicates the amino acid side chain, which varies depending on the acceptor residue.

PARP1, PARP2, PARP5a/TNKS1, PARP5b/TNKS2, and PARP4/vPARP (Kickhoefer et al., 1999; Vyas et al., 2013).

The DNA-dependent PARP enzymes, PARP1 and PARP2 are the most well-studied of the PARPs and are targeted by PARP inhibitors used in clinical practice. PARP1/2 are activated by a variety of aberrant DNA structures, including single strand breaks (SSBs), double stranded breaks (DSBs) and stalled replication forks (Bryant et al., 2009; Durkacz et al., 1980). DNA binding causes allosteric changes in the catalytic domain that allow NAD+ to access the active site (Langelier et al., 2012). Allosteric changes also facilitate PARP1/2 interaction with the cofactor HPF1 to form a composite enzyme active site that is essential for DNA damage-induced ADP-ribosylation (Gibbs-Seymour et al., 2016; Suskiewicz et al., 2020). Upon activation, PARP1/2 are major acceptors for PAR chains, which are linear or branched, and up to 200 units long (Alvarez-Gonzalez and Jacobson, 1987; Lonskaya et al., 2005).

2.2. PAR removal: PARG, TARG1 and ARH3

PARylation is transient because PAR chains are actively degraded by several ADP-hydrolases, of which PARG is the major enzyme (O'Sullivan et al., 2019). A single PARG gene has been identified which encodes five different transcripts generated by alternative splicing (Meyer-Ficca et al., 2004) Three of these isoforms are catalytically active, cleaving the O-Glycosidic bond between ADP-ribose subunits (Fig. 1) (Haince et al., 2006; Hatakeyama et al., 1986). PARG exoglycosidase activity at the termini of PAR chains releases ADP-ribose, whereas PARG endoglycosidase activity releases intact PAR chains (Barkauskaite et al., 2013a; Pourfarjam et al., 2020). However, PARG is unable to remove the final ADP-ribose subunit attached to proteins because of steric hindrance with the acceptor amino acid side chains, and hence other ADP-hydrolases perform this function (Barkauskaite et al., 2013a).

ARH3 and TARG1 are also involved in PAR chain removal (Fig. 1). Like PARG, ARH3 possesses activity towards the O-Glycosidic bond between ADP-ribose subunits, however it has a much lower specific activity against PAR (Oka et al., 2006). The main function of ARH3 in ADP-ribosylation turnover is in the removal of serine-linked oligo- and mono-ADP-ribosylation following DNA damage (Abplanalp et al., 2017; Fontana et al., 2017). In contrast, TARG1 acts predominantly as a mono-ADP-ribose hydrolase on acidic residues (Sharifi et al., 2013). However, TARG1 can also cleave PAR chains between the proximal ADP-ribose and acceptor residue to release full PAR chains, although the significance of PAR release by TARG1 remains unclear (Sharifi et al., 2013; Vyas et al., 2014).

2.3. Parp1/2 and Parg-deficient mouse models

Mice lacking *Parp1* or *Parp2* are viable, but exhibit sensitivity to a variety of DNA damaging agents (Menissier de Murcia et al., 2003). In contrast, dual knockout of *Parp1* and *Parp2* causes embryonic lethality, suggesting PARP1 and PARP2 have partially overlapping functions. Knockout of *Parg* in mice also causes embryonic lethality (Koh et al., 2004). However, trophoblast stem cells derived from the *Parg*-null embryo are viable when cultured in the presence of a PARP inhibitor. Moreover, double *Parg-Parp1* null mice are viable, although they develop severe renal failure (Chen et al., 2019; Koh et al., 2004). Taken together, these observations suggest that firstly, PARylation is essential and secondly, that PARG is the major removal enzyme for PARP1. Due to the embryonic lethality of PARG loss, hypomorphic variants have been used to study PARG function, targeting exons 2 and 3 (*Parg*Δ2/Δ3), leading to loss of the larger catalytically active isoforms (Cortes et al., 2004; Min et al., 2010). Similar to disruption of *Parp1* or *Parp2*, *Parg*Δ2/Δ3 mouse embryonic fibroblasts exhibit elevated sensitivity to DNA damage, highlighting the importance of PARylation to the DNA damage response (DDR) (Min et al., 2010).

3. PARylation in the DNA damage response and DNA replication

PARylation has a role in various biological processes including transcription, mitosis, cell signalling and cell death (Schreiber et al., 2006). PARylation modifies the activity of acceptor proteins and provides scaffolding functions through a network of diverse PAR-binding domains (Barkauskaite et al., 2013b). Rapid removal of PAR returns acceptor proteins back to their basal unmodified state to facilitate completion of biological processes. The roles of PARP1/2 and PARylation within the DDR and DNA replication are the most well-studied, with numerous DDR proteins being targeted by PARylation and possessing PAR-binding domains (Buch-Larsen et al., 2020; Teloni and Altmeyer, 2016). Many PAR-binding domains use electrostatic interactions with negatively charged PAR chains. These interactions can promote PAR-seeded liquid demixing, reorganising the cellular environment to facilitate early stages of DNA repair protein recruitment to DNA break sites (Altmeyer et al., 2015). A corollary of this is that PARG activity prevents excessive accumulation of PAR and various associated proteins, restoring the basal organisation of the cellular environment following DNA repair (Altmeyer et al., 2015; Singatulina et al., 2019). Furthermore, PARylation promotes chromatin relaxation by targeting histones and modulating the activity of chromatin remodellers, such as ALC1, via their PAR-binding domains, in turn facilitating DNA repair (Ahel et al., 2009; Huletsky et al., 1989; Mathis and Althaus, 1987; Poirier et al., 1982).

3.1. PARylation in DNA repair pathways

PARP1/2 are key components of the SSB repair (SSBR) machinery, where PARylation aids the recruitment of repair proteins, in particular XRCC1 (Caldecott, 2008; Durkacz et al., 1980; Fisher et al., 2007). XRCC1 is critical to SSBR to recruit other SSBR components (Caldecott, 2019; El-Khamisy et al., 2003; Whitehouse et al., 2001). PARG is also required for SSBR, and depletion of PARG results in persistent accumulation of PAR-dependent XRCC1 foci indicating that PAR hydrolysis is required for XRCC1 disassembly following repair (Fisher et al., 2007; Gao et al., 2007). Indeed, synthetic lethality between both XRCC1 and PARG, and XRCC1 and PARP has been described highlighting their concerted roles in SSBR (Ali et al., 2018; Martin et al., 2018). In addition, stabilisation of PAR chains attached to PARP1/2 can reduce its association with chromatin (Gogola et al., 2018; Jain et al., 2019; Satoh and Lindahl, 1992), which could interfere with downstream repair events that rely on chromatin-bound PARP1/2 and the recycling of PARP1/2 activity at other lesions.

Another role for PARP1 in DNA repair is in microhomology mediated end-joining (MMEJ). MMEJ is an error-prone method of DSB repair forming an important backup when other repair pathways, such as HR, are impaired (Ceccaldi et al., 2015). PARP1 activation at broken DNA ends can mediate the synapsis and annealing of microhomologous sequences, followed by subsequent recruitment of MMEJ factors including LIG3, and error-prone translesion synthesis (TLS) polymerase, Polθ (McVey and Lee, 2008; Sfeir and Symington, 2015). Extensive autoPARylation of PARP1 impairs its synapsis activity and promotes dissociation of PARP1 and LIG3, suggesting control of PAR stability by PARG could also contribute to this process (Audebert et al., 2004).

PARP1 can also influence DNA resection through recruitment of the nuclease MRE11 (Bryant et al., 2009; Haince et al., 2008). In addition, PARylation can recruit SLX4, which coordinates the nucleases required for Holliday junction resolution during HR (González-Prieto et al., 2015; Rai et al., 2016). However, loss of PARP1 can also lead to hyper-resection of DSBs by the nuclease, EXO1 (Caron et al., 2019), suggesting a complex role for PARP1 in both promoting and protecting against nuclease-mediated resection at DNA breaks.

3.2. Okazaki fragment processing

During replication, DNA synthesis by DNA polymerase on the lagging strand is discontinuous, producing Okazaki fragments that require subsequent processing (Okazaki et al., 1968). This represents a potential source of DNA damage if Okazaki fragment processing is disrupted and unligated fragments accumulate. Inhibiting Okazaki fragment processing induces elevated levels of PARylation and XRCC1 associated at sites of DNA replication, and causes increased sensitivity to PARP1/2 loss (Hanzlikova et al., 2018). This suggests the activation of PARP1/2 by SSBs may be used as a mechanism for sensing unligated Okazaki fragments, and a pathway involving SSBR components may be employed as a back-up for Okazaki fragment processing (Hanzlikova et al., 2018).

3.3. Replication fork stability: fork reversal-restart

When a replication fork stalls in response to stress, the replication fork must be stabilised, and subsequently restarted after the impediment is removed. One mechanism of fork stabilisation is the remodelling into a four-way 'chicken foot' structure known as a reversed fork (Fig. 2). Reversed forks play a protective role in guarding the genome against replication stress, by providing a 'stable' paused structure at the stalled fork and providing an intermediate for restarting replication (Cortez, 2019). Reversed forks have been detected in response to variety of replication stressors and DNA damaging agents, and also under unperturbed conditions (Mutreja et al., 2018; Zellweger et al., 2015), suggesting fork reversal is a global response to replication stress.

Once replication stress has been resolved, replication at reversed forks is re-started through branch migration catalysed by numerous enzymes and the balance of fork reversal and restart is normally transient (Fig. 2) (Berti et al., 2020). The helicase activity of RecQ family member, RECQ1, restores regressed replication forks to facilitate replication restart (Berti et al., 2013). PAR binding to RECQ1 can inhibit its fork restoration activity, stabilising reversed forks (Berti et al., 2013; Ray Chaudhuri et al., 2012). RECQ1 is also PARylated, which may contribute to inhibition of its activity (Iannascoli et al., 2015; Sharma et al., 2012). In this context, PARP1

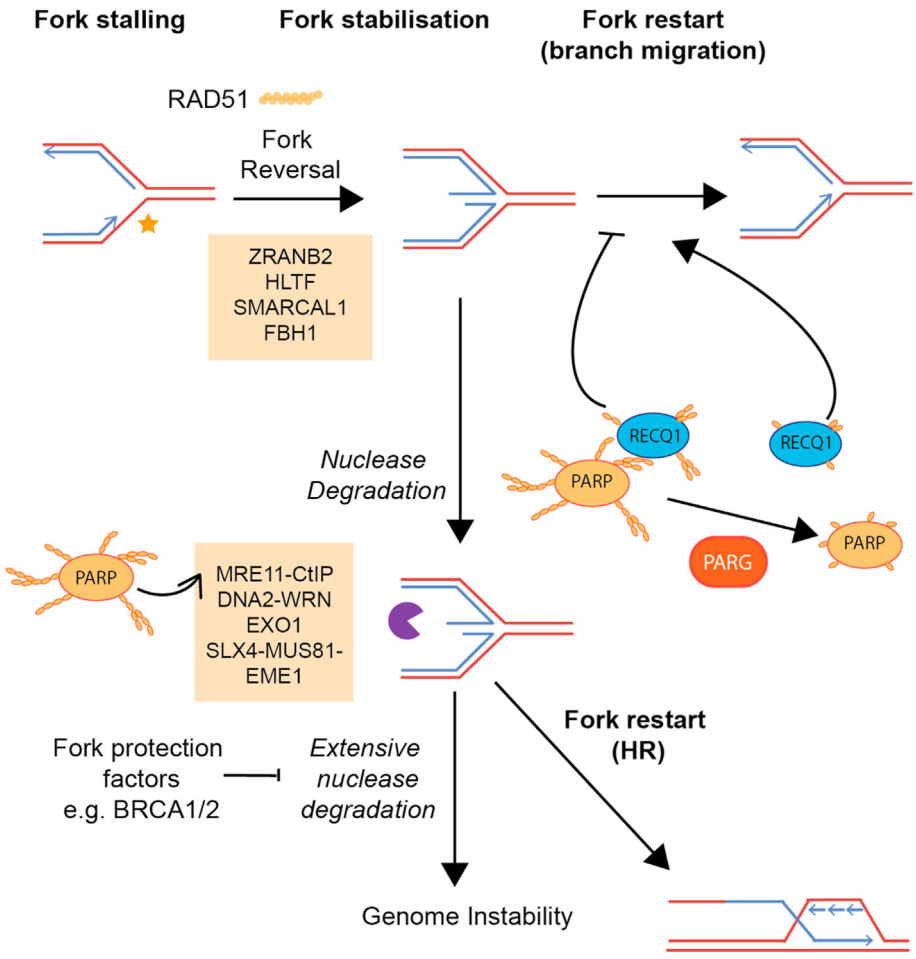


Fig. 2. Pathways of stalled fork remodelling and stability.
When a replication fork stalls fork reversal may be catalysed by a number of enzymes in cooperation with RAD51. Fork reversal can be a transient but frequent transaction at the fork, and fork restart by branch migration can be facilitated by RECQ1. The fork restart activity of RECQ1 is inhibited by the activity of PARP. PARG helps to relieve inhibition of RECQ1 by hydrolysing inhibitory PAR. Reversed forks can be targeted by several nucleases, which can both facilitate HR-mediated restart, or if unregulated lead to extensive degradation and genome instability. Nuclease-mediated degradation is regulated by stabilising RAD51 filaments, such as by BRCA1 or BRCA2.

has a protective function by restraining DNA replication and thus preventing DSB formation through fork run-off at lesions (Ray Chaudhuri et al., 2012). Indeed, PARP1/2 inhibition can lead to elevated fork speed, suggesting the mechanisms that restrain fork progression are disrupted (Maya-Mendoza et al., 2018).

The removal of inhibitory PAR chains by PARG relieves RECQ1 inhibition, maintaining the balance of fork reversal and restart (Fig. 2). Depletion of PARG without additional perturbation causes an accumulation of the normally transient reversed fork structures (Ray Chaudhuri et al., 2015). This suggests inhibition of PARG stabilises PAR chains, reinforcing the inhibition of RECQ1-mediated fork restart, and stabilising reversed forks (Margalef et al., 2018; Ray Chaudhuri et al., 2015).

3.4. Replication fork stability: fork degradation

In addition to fork restart through branch migration, reversed forks also provide an intermediate for fork restart by HR. The nascent DNA at the reversed fork DSB-like end is a substrate for a number of nucleases including MRE11, DNA2 and EXO1, which initiate HR-based fork restart by resection of the DNA (Fig. 2) (Bryant et al., 2009; Lemacon et al., 2017). However extensive end processing by these nucleases can also be detrimental due to accumulation of vulnerable single-stranded DNA (ssDNA) and genome instability (Garzón et al., 2019; Kolinjivadi et al., 2017; Mijic et al., 2017). The junction formed at reversed forks is topologically similar to the Holliday junction formed during HR and can also be cleaved by the coordinated activity of Holliday junction resolvases (Wyatt et al., 2017). Indeed, unscheduled cleavage of the reversed fork structure by SLX4-dependent Holliday junction endonucleases can drive genome instability, by introducing DSBs when fork remodelling is deregulated (Couch et al., 2013).

To protect from excessive degradation, a variety of factors help to modulate accessibility and recruitment of nucleases to stalled or remodelled replication forks (Berti et al., 2020). A key factor in both the formation and protection of reversed replication forks is RAD51 (Hashimoto et al., 2010; Zellweger et al., 2015). Factors that promote RAD51 filament stability also protect the replication fork from nucleolytic degradation, including core HR components BRCA1/2 (Schlacher et al., 2011, 2012). Therefore, in the absence of fork protection factors such as BRCA1/2, unrestrained PARP1-mediated recruitment of nucleases, namely MRE11, can be detrimental (Ding et al., 2016; Ray Chaudhuri et al., 2016). Taken together, both too much and too little fork reversal can be harmful depending on the context. In turn, the cellular context consists of a balance of replication fork protection, reversal, and restart factors.

3.5. Exploiting PARylation in genome stability: PARP inhibitors

Based on the functions of PARylation in the DNA damage response, PARP inhibitors were developed to enhance the cytotoxicity of chemotherapy agents, by interfering with cells ability to repair the DNA damage (Boulton et al., 1995; Fong et al., 2009; Oza et al., 2015). Two seminal studies highlighted the utility of PARP inhibitors as single agents, demonstrating *BRCA1* or *BRCA2* deficient tumours are profoundly sensitive to PARP inhibitors due to a defect in HR (Bryant et al., 2005; Farmer et al., 2005). The original hypothesis proposed is that when PARP1/2 are inhibited, unrepaired SSBs accumulate and are converted into one-ended DSBs at the replication fork (Bryant et al., 2005). During replication, cells are reliant on HR-based repair for DSBs, but in the absence of BRCA1/2, cells are unable to repair via this pathway leading to an accumulation of deleterious DSBs.

It is now known that PARP inhibitor cytotoxicity is not only due to enzymatic inhibition. PARP inhibitors exhibit varying abilities to trap PARP1/2 on DNA, creating a lesion dependent on HR-based repair for removal during DNA replication (Murai et al., 2012; Strom et al., 2011). Based on PARP1/2 functions at replication fork, it is possible that replication fork intermediates, such as Okazaki fragments or regressed forks, could form a source of PARP1/2 binding and trapping.

3.6. Resistance to PARP inhibitors

Multiple mechanisms of PARP inhibitor sensitivity and resistance have been identified and reviewed extensively elsewhere (Lord and Ashworth, 2013; Mateo et al., 2019; Noordermeer and van Attikum, 2019). For example, as PARP inhibitor sensitivity is strongly dependent on HR deficiency, many resistance mechanisms restore HR competency. These include reversion mutations in key HR components (Edwards et al., 2008; Kondrashova et al., 2017; Sakai et al., 2008), or the loss of factors which modulate DNA repair pathway choice, such as REV7 or 53BP1 (Gupta et al., 2018; Jaspers et al., 2013; Nacson et al., 2018; Xu et al., 2015). A HR-independent mechanism of resistance is stalled fork protection. Stalled replication forks are particularly unstable in *BRCA*-deficient cells, owing to the instability of the RAD51 filament, deprotecting the fork from nucleases (Schlacher et al., 2011). This instability can further contribute to PARP inhibitor sensitivity meaning factors that reduce nucleolytic degradation at DNA-PARP lesions associated with the replication fork can cause resistance to PARP inhibitors (Ray Chaudhuri et al., 2016). Thus, the prevalence of PARP inhibitor resistance in the clinic provides a rationale for developing additional therapeutic strategies.

4. PARG inhibitors

4.1. Development of PARG inhibitors

Whilst PARP inhibitors have achieved great success in the clinic, alternative therapeutic strategies are being explored for patients unlikely to benefit from these agents. As timely degradation of PAR chains is critical to the cellular processes that maintain genome stability, PARG inhibitors could also induce selective cytotoxicity of tumour cells. Early attempts to identify small-molecule inhibitors targeting PARG were impeded by limited specificity (e.g. Gallotannin, GPI-16552, RBPI-3) (Falsig et al., 2004; James et al., 2016), and lack of cell permeability (e.g. ADP-HPD); precluding comprehensive evaluation of pre-clinical PARG inhibitor efficacy. The development of the first-in-class selective and cell permeable PARG inhibitor, PDD00017273, led to a plethora of pre-clinical studies investigating its therapeutic potential (Gravells et al., 2017, 2018; Jain et al., 2019; James et al., 2016; Pillay et al., 2019). During characterisation of PDD00017273, a key observation made was that certain cell lines displayed differential sensitivity to PARG versus PARP inhibitors, suggesting that PDD00017273 derivatives could be used to treat a different subset of tumours than PARP inhibitors (James et al., 2016; Pillay et al., 2019). Subsequently, two further compounds targeting PARG have been described, COH34 and JA2131 (Chen and Yu, 2019; Houl et al., 2019), with COH34 also displaying differential sensitivity to PARP inhibition in cell line models. Furthermore, sensitivity to PDD00017273 is absent in non-cancerous stromal or fallopian tube epithelial cells, providing evidence of selective cytotoxicity in tumour cells (Pillay et al., 2019). The three compounds described have different chemical structures, however all three bind the adenine-binding pocket of the PARG catalytic active site, and they are summarised in Table 1.

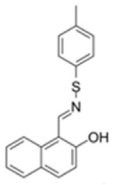
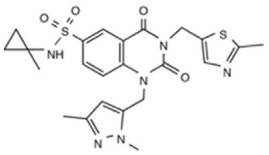
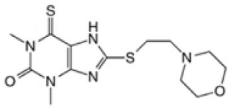
4.2. Sensitisation to damaging agents

Treatment with PDD00017273 and COH34 has been shown to sensitise cells to an assortment of DNA damaging agents, to varying degrees, including those used in the clinic, such as 5-Fluorouracil, temozolomide, and platinum-based agents (Chen and Yu, 2019; Gogola et al., 2018; Jain et al., 2019). Most consistently, treatment with PARG inhibitors causes increased sensitivity to ionising irradiation (Chen and Yu, 2019; Gogola et al., 2018; Gravells et al., 2018; Houl et al., 2019; Jain et al., 2019). This provides evidence for the use of PARG inhibitors as radiosensitising agents, and combinations of PARG inhibitors and ionising radiation may be beneficial in cancers that present with localised disease for which radiotherapy alone is standard of care (e.g. prostate and breast cancer). Of note, several studies have described aberrant mitoses following combined irradiation and PARG inhibition (Amé et al., 2009; Gravells et al., 2018). In contrast, combined irradiation and treatment with the PARP inhibitor olaparib does not appear to affect mitosis (Gravells et al., 2018). This could be related to the ability of PARG to reverse PARylation catalysed by PARP enzymes other than PARP1/2, and the effect of PARG inhibition on cellular processes other than the DDR warrants further investigation (Chang et al., 2004, 2005). In turn, this could reveal novel vulnerabilities targetable by PARG inhibition.

4.3. BRCA1/2 and PARG inhibitor sensitivity

In early studies, *BRCA2* was found to be synthetic lethal with *PARG* using RNAi-mediated depletion, or the non-specific inhibitor of PARG, gallotannin (Fathers et al., 2012). Later studies using the PARG inhibitor, PDD00017273, provided evidence for synthetic lethality between PARG and additional HR-associated factors, including BRCA1, PALB2, FAM175A, and BARD1 (Gravells et al., 2017; Jain et al., 2019). Similarly, sensitivity of *BRCA1/2*-mutant cells to the PARG inhibitor, COH34 has also been described (Chen and Yu, 2019). Inhibition of PARG likely generates replication fork-associated lesions that are dependent on HR for repair. One possibility is that when PARG is inhibited, reversed forks accumulate and cells become more dependent on BRCA1/2 for HR-mediated fork restart. Alternatively, inhibiting PARG may interfere with SSBR, leading to DSBs at replication forks that require HR for repair and restart, as described for PARP inhibition (Bryant et al., 2005; Fisher et al., 2007). However, there is some conflicting evidence for *BRCA1* deficiency in determining sensitivity to PARG inhibition, which could suggest *BRCA1* deficiency is not sufficient for sensitivity to PARG inhibition (Jain et al., 2019; Noll et al., 2016). Moreover, several studies have demonstrated PARG inhibitor sensitivity can occur in *BRCA* wild-type, or HR-proficient cells, highlighting the role of other molecular determinants of PARG

Table 1
Table comparing cell active, selective PARG inhibitors discussed in the text. *Indicates original characterisation study. TR-FRET- Time-resolved fluorescence energy transfer.

Studies	Compound	Screening Method	Compounds Screened	Derivative	Structure
Chen et al. (2019b)	COH34	*In silico*	290,000	Napthol	
James et al. (2016)*; Gravells et al. (2017, 2018); Jain et al. (2019); Gogola et al. (2018); Hanzlikova et al. (2018); Pillay et al. (2019)	PDD00017273	TR-FRET based	1,400,000	Quinazolinedione	
Houl et al. (2019)	JA2131	TR-FRET based	1990	Thio-methylxanthine	

inhibitor sensitivity (Houl et al., 2019; James et al., 2016; Pillay et al., 2019).

4.4. Replication factors and PARG inhibitor sensitivity

Several components of the replication machinery were identified as synthetic lethal with PARG, using PDD00017273, namely HUS1, RFC2 and TIMELESS (Pillay et al., 2019). Disrupting these factors interferes with fork progression, replication checkpoint signalling and fork recovery (Smith et al., 2009; Técher et al., 2017). Checkpoint defects can destabilise replication forks further, by deregulating fork remodelling and the nucleolytic processing of replication intermediates (Couch et al., 2013). It is possible that in this context of elevated fork stalling, cells become more dependent on PARG-regulated fork progression and restart. In particular, TIMELESS depletion induces PARG inhibitor sensitivity in multiple ovarian cancer cell lines, indicating a robust PARG-TIMELESS synthetic lethal relationship (Pillay et al., 2019). Moreover, TIMELESS deficiency can render cells sensitive to PARG, but not PARP inhibition, highlighting the potential for targeting genotypes with PARG inhibitors that do not benefit from PARP inhibitors (Pillay, N. and Brady, R.M., unpublished results).

4.5. PARG inhibitor-induced replication catastrophe

Studies using newly developed PARG inhibitors, PDD00017273 and JA2131, have demonstrated PARG inhibitor treatment impairs replication fork progression and increases sensitivity to pharmacological induction of replication stress (Gravells et al., 2017; Houl et al., 2019; Pillay et al., 2019), corroborating previous studies based on the genetic perturbation of PARG (Illuzzi et al., 2014; Min et al., 2010; Ray Chaudhuri et al., 2015). Persistent replication stress leads to an accumulation of ssDNA and exhaustion of the ssDNA binding factor, RPA, causing replication catastrophe (Toledo et al., 2013). Following PDD00017273 treatment of sensitive cells, an S-phase dependent accumulation of nuclear RPA is observed, coincident with high levels of pan-nuclear γH2AX staining, which are phenotypes associated with replication catastrophe (Pillay et al., 2019; Toledo et al., 2013). An earlier study using RNAi also observed the pan-nuclear γH2AX staining pattern using a combination of PARG knockdown and prolonged treatment with the ribonucleotide reductase inhibitor, hydroxyurea (Illuzzi et al., 2014). In contrast, the authors found RPA recruitment was impaired when PARG was depleted (Illuzzi et al., 2014). It was demonstrated that RPA is targeted for PARylation, and detrimental accumulation of PAR can inhibit RPA binding to DNA. This could exacerbate replication catastrophe by preventing ssDNA protection. Indeed, mass spectrometry datasets confirm RPA is a target for ADP-ribosylation following stress (Buch-Larsen et al., 2020). The discrepancies in RPA recruitment between these studies could be based on the use of hydroxyurea, which may induce higher levels of PARylation relative to unperturbed conditions.

The replication catastrophe model postulates that RPA exhaustion is a prerequisite for fork breakage and collapse *en mass* (Toledo et al., 2017). However, PARG inhibition stabilises reversed forks as RECQ1 fork restoration is inhibited, which limits the accumulation of ssDNA at stalled forks (Ray Chaudhuri et al., 2015). Therefore, nuclease-mediated degradation is necessary to produce ssDNA and RPA accumulation at these structures (Fig. 3). Nuclease-mediated degradation can also directly cause DSBs and fork collapse at reversed forks (Couch et al., 2013). Accordingly, the accumulation of chromatin-bound RPA and broken forks is likely coupled, as opposed to forming sequential events. In support of this, we find MRE11 inhibition suppresses the accumulation of nuclear RPA and pan-nuclear γH2AX induction in PARG inhibitor-sensitive cells (Pillay, N., unpublished results). Regardless of the role of RPA exhaustion in this context, excessive fork collapse and DNA breakage likely leads to a 'point of no return' that prevents cell recovery and correlates with the distinctive pan-nuclear γH2AX staining pattern. Any context that increases the number of stalled forks in a cell would be expected to increase the number of forks that reverse and collapse with PARG inhibition and are thus more likely to approach the threshold for irreversible damage.

4.6. A gene expression signature for PARG inhibitor sensitivity

Our observations indicate that there are differences between how cell lines respond to single-agent PARG inhibition, which indicates that there are specific molecular determinants driving sensitivity (Pillay et al., 2019). Given that depletion of replication

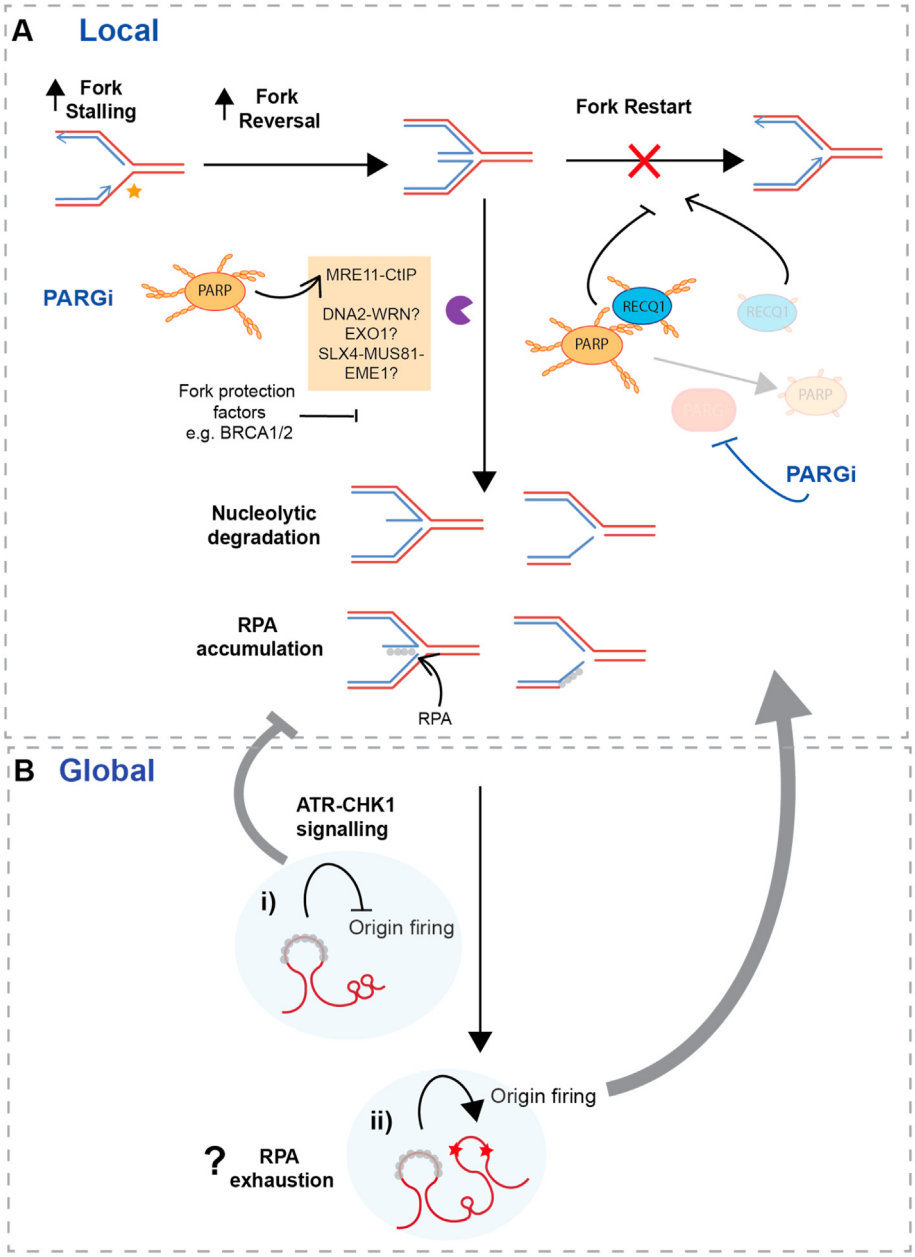


Fig. 3. Models for sensitivity to PARG inhibition.
(A) Increased replication stress increases the frequency of fork stalling. When PARG is inhibited, PAR chains are stabilised and RECQ1 fork restoration is inhibited. This stabllises reversed forks, which can be targeted by various nucleases, such as MRE11. MRE11 recruitment may be facilitated by PARylation. MRE11 can act at reversed forks to cause ssDNA accumulation. Other nucleases can directly cleave the regressed fork to induce fork breakage. ssDNA is bound and protected by RPA. (B) In i) RPA activates the ATR-CHK1 axis which inhibits global origin firing, preventing the formation of additional stalled and reversed forks. In ii) eventually global origin firing ensues leading to the same sequence of events in (A), RPA sequestration and eventual RPA exhaustion. Exposure of vulnerable ssDNA causes excessive DNA damage. Note, direct cleavage of reversed forks can also contribute to damage formation and the contribution of RPA exhaustion to fork breakage remains to be fully established (?).

factors can induce PARG inhibitor sensitivity, we hypothesised that underlying differences in replication factor levels could explain the differences in PARG inhibitor response between cell lines. Gene expression analysis indicates that cell lines that respond to PARG inhibition exhibit lower expression levels of genes involved in DNA replication, relative to PARG inhibitor-resistant cell lines (Coulson-Gilmer et al., 2020; Pillay et al., 2019). Therefore, one hypothesis is that the levels of replication components could determine dependence on PARG activity; for example, cells with lower levels of core replication components may be less able to tolerate oncogene-induced replication stress, and experience increased levels of fork stalling, that subsequently require PARG-regulated fork reversal and restart (Bianco et al., 2019).

4.7. PARG inhibitor combination with replication checkpoint inhibitors

Several small molecule inhibitors targeting CHK1 are in clinical development and have been assessed as monotherapy, and in combination with other traditional DNA damaging cytotoxic agents, such as cisplatin, cyclophosphamide and gemcitabine. A novel targeted therapeutic approach may involve combining CHK1 and PARG

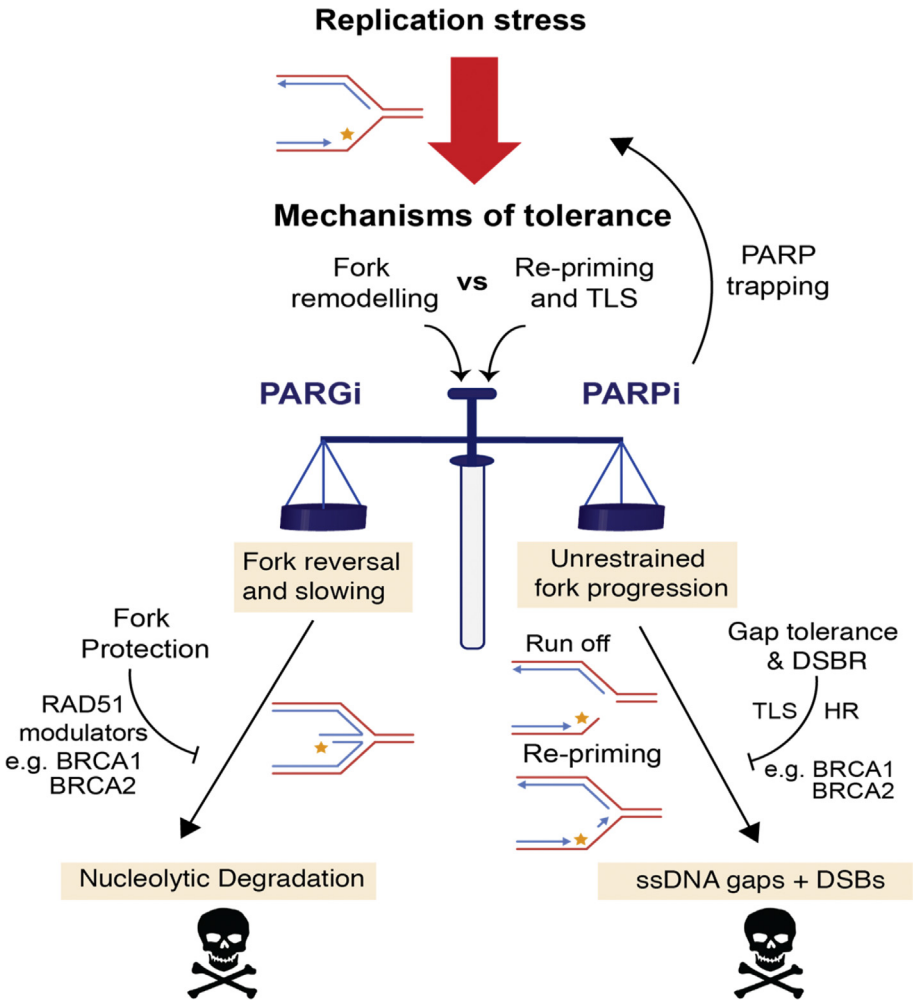


Fig. 4. Sensitivity to PARG versus PARP inhibitors.
Replication stress promotes fork remodelling into a reversed replication fork and is regulated by a number of factors. PARG inhibition (PARGi) enhances the accumulation of reversed forks. Various proteins also contribute to protection of the reversed replication fork from nucleases, many of which modulate RAD51 stability, such as BRCA1 and BRCA2. If the balance of fork remodellers favours reversal, reversed forks may be more likely to form. In addition, loss of fork protection may enhance nucleolytic degradation causing toxicity of PARGi. Conversely, treatment with PARP inhibitors (PARPi) promotes unrestrained fork progression, and PARP trapping exacerbates replication stress by producing fork stalling lesions. Unrestrained fork progression in the presence of stress such as trapped PARP may lead to fork run-off and DSB formation at the fork. Alternatively, repriming by PRIMPOL can cause ssDNA gaps. Both DSBs and ssDNA gaps must be repaired for cellular viability, which includes repair by HR or translesion synthesis (TLS) at the ssDNA gap. Cells lacking these mechanisms may be sensitive to PARPi.

inhibition, which results in enhanced cytotoxicity in several ovarian cancer cell lines and *ex vivo* patient-derived ovarian cancer models (Pillay et al., 2019). In contrast, tumour matched non-cancerous stromal cells are resistant to this combination, suggesting cancer-specific vulnerabilities, such as oncogene-induced replication stress, could confer sensitivity. Another important observation was that sensitivity to CHK1 + PARG inhibition differed from sensitivity to CHK1 + PARP inhibition in certain contexts, which is of interest given the CHK1 and PARP inhibition combination is undergoing pre-clinical evaluation (Kim et al., 2017; Pillay et al., 2019). This difference suggests that the combination could be used to sensitise distinct genotypes from those sensitive to PARP inhibition. Furthermore, loss of PARG is a mechanism of resistance to PARP inhibitor treatment, which could form an acquired vulnerability to exploit with CHK1 inhibition (Gogola et al., 2018).

The concerted functions of ATR and CHK1 at the checkpoint, and the ongoing development of ATR inhibitors, provides a rationale for also testing the PARG + ATR inhibitor combination (Buisson et al., 2015). With the recent commercial availability of the three newly described compounds targeting PARG, PDD00017273, COH34 and JA2131, a direct comparison of their efficacies will be paramount, as single agents and combination therapies in a range of pre-clinical models.

5. Sensitivity to PARP versus PARG inhibition

PARP and PARG are antagonistic enzymes and therefore inhibition often has opposite effects. PARP inhibitors prevent the formation of PAR chains whereas PARG inhibitors stabilise PAR chains. Indeed, restoring the balance of PARylation by reducing the activity of the reverse enzyme can mediate resistance to PARP or PARG inhibitors (Gogola et al., 2018; Pillay et al., 2019). At the replication fork, the response to replication stress is controlled by this delicate balance of PAR turnover. PARP inhibition promotes unrestrained fork progression whereas PARG inhibition halts fork progression by stabilising reversed forks, both of which can be detrimental. The molecular context of a cell consisting of a balance of various fork stability components likely determines whether a cell is sensitive to PARP inhibitors, PARG inhibitors, or both (Fig. 4).

We propose that two factors are required for PARG inhibition to become toxic to cells; 1) replication stress to increase the frequency of stalled and subsequently reversed forks and; 2) exposure to excessive nucleolytic degradation. Factors which reduce fork reversal or nuclease-mediated degradation could mediate resistance; for example, the enzyme PrimPol can re-prime DNA replication ahead of fork impediments and therefore it is a central factor to opposing fork reversal (Garcia-Gomez et al., 2013; Mouron et al., 2013). It has been demonstrated that PrimPol can mediate resistance to replication stress by limiting the accumulation of toxic reversed forks (Bai et al., 2020; Quinet et al., 2020). Thus, if toxic-reversed forks are the cause of PARG inhibitor sensitivity, PrimPol could mediate resistance to PARG inhibition.

Conversely, inhibition of PARP promotes unrestrained fork progression. This can introduce DSBs through fork collision with lesions, and ssDNA gaps through re-priming mechanisms or unprocessed Okazaki fragments, both of which require repair to prevent toxicity (Fig. 4) (Cong et al., 2019; Hanzlikova et al., 2018; Ray Chaudhuri et al., 2012). This is exacerbated by PARP trapping onto DNA, increasing the dependency on replication-associated repair mechanisms.

6. Concluding remarks and future perspectives

In this review we have discussed the developing views of PARylation in controlling replication fork progression. The molecular details of how PARylation integrates signalling at the replication fork, and the targets of PARylation at the replication fork, remain largely unknown. PARG inhibitors will provide a tool to study the regulation of replication fork processes by PARylation, since these reactions are transient and difficult to study (Hanzlikova et al., 2018). In turn, this will develop our understanding of the mechanisms of action of PARG and PARP inhibitors.

It is evident that replication stress is a vulnerability that can be exploited by PARG inhibition (Illuzzi et al., 2014; Pillay et al., 2019). Defining the nature of this vulnerability will be important to establishing guided therapeutic strategies using PARG inhibitors by; a) developing a 'replication stress' gene signature that can predict PARG inhibitor sensitivity, b) testing synergy between PARG and ATR/CHK1 inhibitors in a range of pre-clinical models, and c) conducting genome-wide screens to find additional determinants of PARG inhibitor sensitivity, that can be used to predict sensitive tumours.

Whilst PARP inhibitors have been a huge success story, many patients diagnosed with ovarian cancer are unlikely to benefit from these agents and resistance often develops. PARP and PARG inhibitors display differential sensitivity in cell line models, and PARG inhibitors show efficacy in *BRCA* wild-type cells (Houl et al., 2019; James et al., 2016; Pillay et al., 2019). Thus, PARG inhibitors hold promise in targeting distinct tumour genotypes to PARP inhibitors in the clinic, and clinical studies should focus on utilising PARG inhibitors in patients less likely to respond to PARP inhibitors.

Author statement

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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