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Clinical use and mechanisms of resistance for PARP inhibitors in homologous recombination-deficient cancers

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ABSTRACT

Cells are continuously subjected to DNA damaging agents. DNA damages are repaired by one of the many pathways guarding genomic integrity. When one or several DNA damage pathways are rendered inefficient, cells can accumulate mutations, which modify normal cellular pathways, favoring abnormal cell growth. This supports malignant transformation, which can occur when cells acquire resistance to cell cycle checkpoints, apoptosis, or growth inhibition signals. Mutations in genes involved in the repair of DNA double strand breaks (DSBs), such as *BRCA1, BRCA2,* or *PALB2*, significantly increase the risk of developing cancer of the breast, ovaries, pancreas, or prostate. Fortunately, the inability of these tumors to repair DNA breaks makes them sensitive to genotoxic chemotherapies, allowing for the development of therapies precisely tailored to individuals' genetic backgrounds. Unfortunately, as with many anti-cancer agents, drugs used to treat patients carrying a *BRCA1* or *BRCA2* mutation create a selective pressure, and over time tumors can become drug resistant. Here, we detail the cellular function of tumor suppressors essential in DNA damage repair pathways, present the mechanisms of action of inhibitors used to create synthetic lethality in *BRCA* carriers, and review the major molecular sources of drug resistance. Finally, we present examples of the many strategies being developed to circumvent drug resistance.

Introduction

Mammalian cells are exposed daily to a variety of endogenous and exogenous genotoxic agents. Exposure to radiation and chemicals of natural or human origin introduces breaks or modifications in the DNA. These need to be repaired in a timely fashion to avoid mutations from being passed on to daughter cells following DNA replication and cell division. Several distinct mechanisms recognize DNA damage, process DNA ends or modified bases, and ensure restoration of the correct genetic information (Fig. 1).

Out of all types of DNA damage, two events can be considered most deleterious for human cells: the double strand break (DSB), and replication fork collapse (RFC). If not properly repaired, both can lead to extensive loss of genetic material, genome rearrangement, and ultimately cancer.

Homologous recombination (HR) and Non-Homologous End Joining (NHEJ) are the two major pathways to repair DSBs. HR is considered more faithful than NHEJ, as it involves extensive homology search prior to repair. Conversely, NHEJ requires little homology, and ensures the ligation of DNA ends at the break. While NHEJ is operational throughout most of the cell cycle, homologous recombination is only effective during the S and G_2 phases of the cell cycle, limiting its utility [1,2]. Since eukaryotes rely heavily on NHEJ to repair DSBs, efficient mechanisms are in place to limit errors and increase fidelity of the NHEJ [3–5]. However, NHEJ is still considered error-prone, as it results in frequent deletions/insertions (indels), and for this reason can be used with high efficiency to create targeted mutations using the CRISPR-Cas9 system.

Interestingly, while HR is considered faithful, mutations in HR proteins and inefficient HR are a major cause of genomic instability and are responsible for many familial cancer predispositions. Efficient DNA damage repair relies on a complex and well-orchestrated sequence of events. Upon DSB, the damage is sensed and signaled, cell cycle checkpoints are activated, and DNA damage repair (DDR) pathways are activated [6]. HR is initiated with resection of the DSBs to create 3′ single-stranded DNA (ssDNA) strands that are essential for HR and inhibit NHEJ. The recombinase RAD51 is a DNA strand-exchange protein

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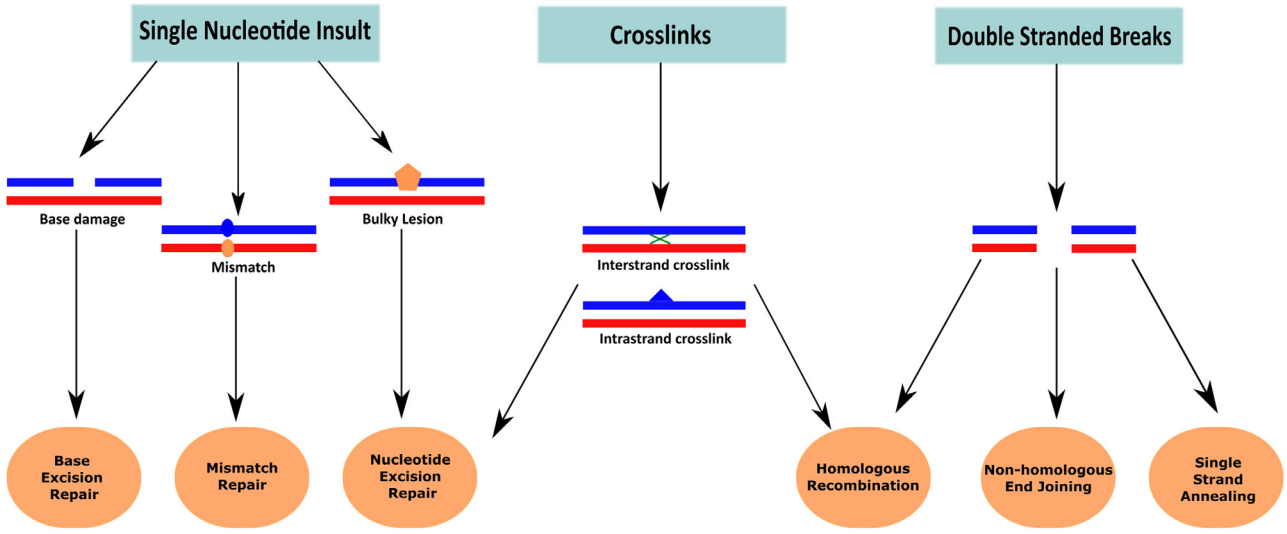

Fig. 1. DNA damages and their major repair mechanisms. Several different types of DNA damage can be discerned. Depending on the type of damage and the cellular and molecular context, they can be repaired via various different DNA repair mechanisms.

that provides the defining step of HR, by binding to the ssDNA overhang, forming a structure named the presynaptic filament [7]. RAD51 bound to DNA is responsible for homology search, duplex capture, invasion and strand exchange [8,9]. Utilizing the sister chromatid within the homologous duplex, restoration of the original DNA sequence prior to the damage is possible [10]. RAD51 cannot carry this complex sequence of events without help from mediator proteins [11–14], which facilitate all steps of HR. Sequencing and mutation analysis *in vivo* and *in vitro* have demonstrated that the complex formed by three tumor suppressors BRCA1, PALB2, and BRCA2 plays an essential role in HR.

BRCA1 functions in the process of HR by promoting resection of DSBs to create 3′ ssDNA overhangs. The BRCA1-PALB2-BRCA2 complex is critical for the strand exchange reaction and loading of the RAD51 recombinase protein onto the ssDNA to create the presynaptic filament [15]. Genetically engineered mutations in RAD51 phenocopy BRCA2 mutations. While cells with defective *BRCA1, BRCA2*, or other HR genes accumulate mutations at a high rate, they also exhibit exquisite sensitivity to radiation, platinum-derived drugs that crosslink the DNA, or to any other form of DNA damage that induces DSBs directly or indirectly. In recent years, molecules that target DNA repair pathways other than HR have been developed to specifically kill HR-deficient cells. All adducts, base modifications and single stranded DNA breaks (SSBs) can eventually become DSBs upon passage of the replisome, in the DNA synthesis phase of the cell cycle. For this reason, increasing the lesion burden, blocking their repair, or causing the stalling and collapse of replication forks all increase DSBs, which cannot be repaired in a *BRCA1/2* deficient background. Apoptosis then occurs. Of note, the use of principle, referred to as 'synthetic lethality', is extremely attractive for patients carrying a *BRCA*-deficiency, as they often inherit one mutated allele from a parent, and are heterozygous for the *BRCA* gene in all cells but the tumor. This potentially allows usage of low doses of chemotherapy and limits side effects for healthy cells. While HR deficiency can be exploited to design efficient therapeutic courses tailored to *BRCA1/2* mutation carriers, such cancers are extremely unstable, commonly recur, and prone to drug resistance.

In this review, we present an overview of the links between DNA damage repair machineries and cancer; we detail the mode of action of PARP inhibitors and their potential role in non-*BRCA* cancers. We then review the complex mechanisms that make a cell resistant to therapy, and finally present recent advances being developed to counter drug resistance.

DNA damage repair and familial cancer

Frequently mutated DNA damage repair pathways

Homologous recombination

Mutations in the *BRCA1* and *BRCA2* genes are associated with an increased lifetime risk for breast and ovarian cancers. The lifetime risk for breast cancers in women with mutations in either *BRCA1* or *BRCA2* is 45–80%, while the lifetime risk for ovarian cancer in *BRCA1*-mutation carriers is 45–60% and 11–35% for *BRCA2*-mutation carriers [16]. While mono-allelic inactivation of these tumor suppressors leads to cancer predisposition, biallelic inactivation of *BRCA1, BRCA2*, or their partner *PALB2*, causes the rare Fanconi Anemia (FA) syndrome [17–19]. Often found in 'triple-negative' breast cancer tumors, so called due to their lack of expression of the estrogen receptor (ER), progesterone receptor (PR), and HER2 receptor, *BRCA1* mutations are predominantly associated with basal-like subtypes of breast cancers. As 70% of *BRCA1* mutant breast cancers lack ER expression [20], this makes them difficult to treat. BRCA2-associated breast cancers, however, are usually ER-positive and p53-negative [21].

The *BRCA1* gene is located on chromosome 17q21 and encodes a multi-functional protein that is involved in DNA damage repair, cell-cycle arrest, transcriptional activation, tumorigenesis and genetic instability [22–24]. Mutations in BRCA1 are mostly observed in three particular domains, the N-terminal RING domain, exons 11–13 and the C-terminus. The RING domain interacts with the protein BARD1 and plays a key role in the ubiquitination pathway during S phase of the cell cycle [25–27]. Exons 11–13 are critical for the tumor suppressor function of BRCA1 [28,29]. The C-terminal domain plays a key role by interacting with various substrates of the DNA damage-activated kinases ATM and ATR, as well as transcription regulators, such as p53 and damage repair proteins, such as CCDC98/CtIP [30,31].

The *BRCA2* gene is also highly involved in much of the same processes as *BRCA1* and consists of 27 exons with eight internally repeated sequences called BRC motifs that are essential for RAD51 interaction, and thus for HR [32,33].

While *BRCA1* and *BRCA2* are probably the most frequently mutated HR genes in familial cancer and increase risk for cancer in the breast, ovaries, pancreas, lung, prostate, and other organs, these are not the only HR genes conferring predisposition to cancer. *ATM, PALB2, CHK2, BARD1, RAD51C* to cite a few, are also essential players of HR and have been found mutated in cancer (Table 1). Cells deficient for repair by HR

Table 1
DNA repair genes deregulated in familial cancer, and their penetrance in breast cancer.

Gene[a]	Function/Pathway	Penetrance[b]
AKT1	AKT signaling	Low
ATM	Double Strand Break (DSB) repair	Intermediate
BARD1	BRCA1-associated protein complex	Intermediate
BRCA1	BRCA1-associated protein complex	High
BRCA2	Fanconi/BRCA	High
BRIP1	Fanconi/BRCA	Intermediate
CDH1	Cell adhesion	High
CHEK2	DSB repair	Intermediate
EXO1	DSB repair	Unknown
FAM175A/Abraxas	DSB repair	Intermediate
GEN1	DSB repair	Unknown
MRE11/RAD50/NBS1	DSB repair	Intermediate
PALB2	Fanconi/BRCA	Intermediate
PIK3CA	AKT signaling	Unknown
PTEN	PI3K/MAPK Signaling	High
RAD51	DSB Repair	High
RAD51C	Fanconi/BRCA	Intermediate
RAD51D	Fanconi/BRCA	Intermediate
STK11	Cell Cycle/p53 regulation	High
TP53	Cell growth	High
XRCC2	DSB repair	Intermediate

[a] Some of the major DNA repair genes found mutated, silenced, or over-expressed in familial breast cancer.
[b] Penetrance is indicated as low (<5%) intermediate (5–20%) or high (>20%) increased lifetime risk of developing breast cancer.

and exhibiting typical *BRCA*-deficient phenotypes, such as sensitivity to DSB-creating drugs, are often referred to as exhibiting "BRCA-ness". While the exact criteria for BRCA-ness have not been defined, gene signatures and major genome rearrangement events have been identified in whole-genome sequencing studies and this can help profile patient samples and predict their response to genotoxic chemotherapy [34–37].

Other DNA repair pathways

For the purpose of this review, we will focus on HR-deficient cancers and their treatment with PARPi and platinum-derived drugs. However, one cannot ignore the essential role other DNA repair mechanisms play to prevent cancer development, by limiting mutations and maintaining genomic stability. For example, DNA mismatch repair is linked to colon cancer suppression [38,39]. Breast, gastric, and colorectal cancers are all suppressed by Base Excision Repair mechanisms (BER), while Nucleotide Excision Repair is essential for the removal of UV-damaged nucleotides and cross-link repair, thus limiting the risks of skin melanoma [40–43].

In addition, it has recently become evident that another, more discreet attack on the DNA can be as deleterious and oncogenic as external genotoxic substances, if not more. Endogenous increase of replication stress, as defined by a difference in the replication fork processivity, excessive firing of origins, or stalled forks that cannot be rescued, is a major source of genomic instability and carcinogenesis [44]. Accumulation of SSB or adducts in the DNA, a deficient replisome, which processivity and/or accuracy is not fully controlled, and improper firing of origins of replication, are some of the mechanisms that can increase the risk of fork stalling. Inactivation of any of the many proteins protecting the fork against extensive resection, or promoting its restart over collapse, all contribute to increased replicative stress. Early stages of carcinogenesis often exhibit high levels of replicative stress and lack of cell cycle checkpoints, and for years genotoxic chemotherapy has been exploiting the fast growth of cancer by enhancing replicative stress.

Following major advances in our understanding of human replication mechanisms and their coupling with DNA repair machineries, therapies are now being specifically designed to target endogenous replication stress, especially those caused by identified mutations in tumor suppressors and oncogenes [45,46].

PARP1, PARP2, and PARG

Poly (ADP-ribose) polymerases (PARPs) are enzymes that are important for the cellular response to single-stranded DNA damage [47]. Specifically, PARP1 and PARP2 are recruited to sites of DNA damage via signals from unligated Okazaki fragments [48] and become activated when binding to the regions of DNA damage. Upon activation, PARP uses DNA nicking to recruit other repair proteins and subsequently uses energy from NAD+ to add polymers of PAR to itself and the other repair proteins. This process, referred to as PARylation [49,50], leads to the generation of a repair complexes that mend the damaged DNA and allows the cell to survive. The degradation of PAR, also known as dePARylation, is an important step in allowing the repair process to proceed after the repair complex has been formed. This process is mediated by a molecule known as poly (ADP-ribose) glycol-hydrolase (PARG) [48,51]. Accumulation of unrepaired DNA insults has dire consequences for the human genome and its stability, and eukaryotic cells have evolved redundant mechanisms to survive the loss of critical functions. Interestingly, while the loss of PARG in mouse cells increases sensitivity to cellular stress [52], it may lead to PARP-inhibitor resistance via the permission of the accumulation of PAR in BRCA2-deficient cells, highlighting the role of PARG molecules in facilitating DNA repair processes [48]. The many mechanisms of drug resistance will be detailed in a later section.

Exploiting DNA repair deficiencies for medicine

Synthetic lethality and PARP inhibition: a success story for HR deficient cancers

HR-deficient cells are extremely prone to accumulation of mutations, loss of genetic information, and genome rearrangement. As a result, these cells are at a high risk of losing tumor suppressors and growth inhibitions, and undergo tumorigenesis. Such cancers can be difficult to treat for many reasons. First, inherited mutations predispose one to early onset cancers. Early onset cancers are generally more advanced cancers at diagnosis [53], which are harder to treat than early-stage cancers. Second, the genome of HR deficient cells, such as cells from *BRCA1/2* mutations carriers, are unstable, and this can give rise to a variety of individual clones of heterogenous genetic background. Last, the penetrance of inherited mutations is such that primary tumors can arise rarely concomitantly or more often sequentially in various tissues within the same patient [54–56], dramatically complicating diagnosis and treatment. Interestingly, these tumors, which can be aggressive in a patient, are often difficult to culture *in vitro*. This stems from their heightened sensitivity to exogenous stressors and their heavy reliance on biological molecules, such as specific growth factors and hormones. Moreover, their incapacity to repair DSBs makes them very sensitive to radiotherapy *in vitro* and in animal models, and genotoxic chemotherapy *in vitro* and in clinical settings. Inactivating a second DNA repair pathway ensures active and efficient killing of cells homozygous for *BRCA1/2* mutations. The best example of this strategy has been the development of PARP inhibitors and their approval in the clinics. Most clinically relevant PARP-inhibitors work by binding to activated PARP1 molecules and trapping them onto damaged DNA [57] leading to stalling of the replication fork and the accumulation of single-stranded DNA breaks (SSBs). PARP inhibition also deregulates fork speed [58] and stability. Last, PARP inhibition can favor NHEJ above HR, which causes increased mutation rates. These unrepaired SSBs progress into double-stranded breaks (DSBs) upon passage of the replication fork. While cells that have intact HR are capable of repairing these DSBs that are created by PARPi in S-phase, cells with mutations in *BRCA1, BRCA2* or *PALB2* are unable to effectively perform HR, promoting the persistence of these DSBs [59–61]. As a result, *BRCA1*- or *BRCA2*-deficient cells have traditionally demonstrated synthetic lethality with the use of PARP-inhibitor treatment, a concept defined as causing cell death with the combination

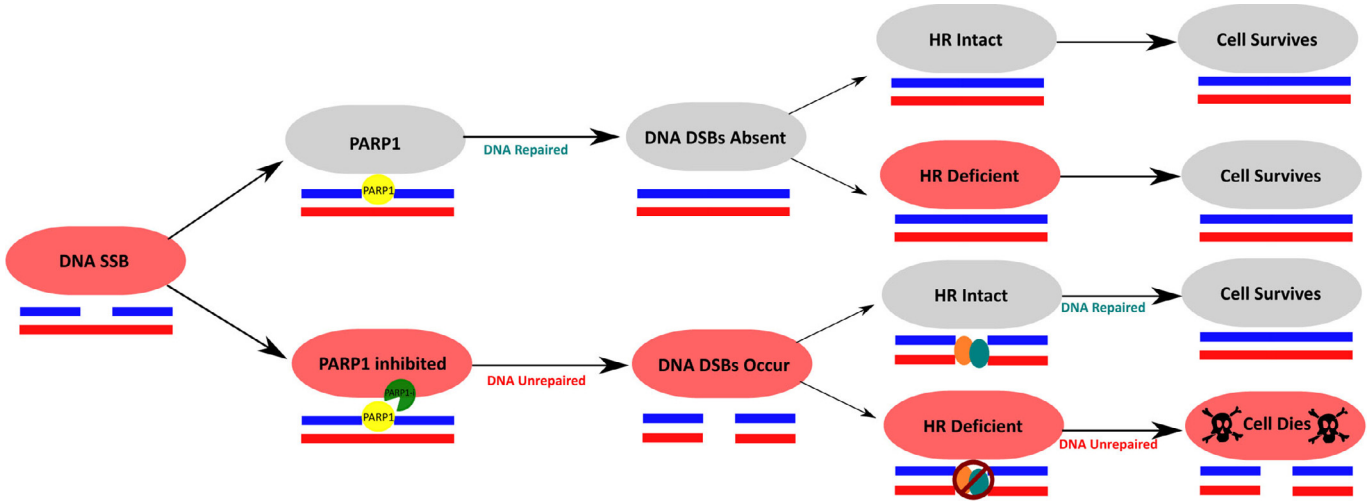

Fig. 2. PARPi exploits HR deficiency to induce synthetic lethality. Concomitant HR deficiency and inhibition of PARP using PARP inhibitors (PARPi) causes cell death, while on their own, neither leads to cell death.

of two defects, here simultaneous HR deficiency and PARP inhibition, but cell survival when each occurs alone [57] (Fig. 2).

Ultimately, cancer cell death is the goal of PARPi treatment, and thus *BRCA1/2* deficient patients have been the primary target population for treatment with this class of drugs. PARPi also shows promises in treating carriers of a *PALB2* mutation, and has been tested with some success on triple-negative breast cancers, serous carcinomas of the ovaries, tumors with high replicative stress levels, and in combination with immune checkpoint blockade [62–66]. This cautiously offers hope for the development of novel combination therapies. Currently, three third generation PARPi molecules have been approved for the treatment of BRCA1/2 deficient breast and ovarian cancers: olaparib, niraparib, and talazoparib. A fourth, veliparib, is under investigation for the treatment of breast, lung, and ovarian cancer. In the clinic, PARPi have been mostly successful, but also exhibited a range of efficacy based on the molecule used and the cancer treated. This is likely due to slightly different mechanisms of action of the various inhibitors, and the way they impact the PARPi-DNA complex, specifically, whether they trap or exclude PARP1 to make it non-functional. The full extent of these mechanism has only started to be understood, thanks to elegant and recent functional [67] and structural studies [68].

PARPi combination therapies for HR proficient cancers

Pin1 inhibition via ATRA

Pin1 is a molecule that has been found to stabilize BRCA1 and inhibition of Pin1 increases sensitivity to PARP-inhibitor treatment, causing a near-complete block in cell proliferation [69,70]. All-trans retinoic acid (ATRA), a drug that is well-known for its treatment in acute promyelocytic leukemia, has been found to have Pin1 inhibitory effects. Combining ATRA with olaparib has been shown to decrease tumor growth and induce apoptosis in *BRCA1/2* proficient triple-negative breast cancer cells. ATRA has the potential to be used to disrupt homologous recombination and sensitize cancer cells to treatment with PARP-inhibitors. Studies to assess the toxicity of the combination therapy are warranted.

Alantolactone

Another agent that can be used to induce PARP-inhibitor sensitivity in HR-proficient cells is alantolactone, via its mechanism of inhibition of thioredoxin reductase leading to selective accumulation of oxidative DNA damage in cancer cells [71]. Of note, this particular drug sensitizes cancer cells to PARPi that induce PARP trapping. Veliparib is a PARP-inhibitor that causes limited DNA damage signaling and lacks potent ability to cause PARP trapping, and thus its combination with alantolactone does not produce synergistic effects. However, combination of alantolactone with olaparib, a strong PARP-trapping drug, has been shown to induce significant synergistic effects. This finding has the potential to broaden the cancer population that can be treated with PARP-inhibitors [72]. The combination of the two drugs requires further assessment in clinical trials.

CDK12 inhibitors

CDK12 is thought to be involved in promoting the expression of DNA-damage response genes such as *BRCA1, ATR, FAN1,* and *FANCD2* [73]. Inhibition of CDK12 with the drug dinaciclib has the potential to sensitize cells to PARP-inhibitors in both *BRCA*-deficient and *BRCA* wild type triple-negative breast cancer cells. It probably does so by promoting genomic instability and increase sensitivity to DNA damage [74]. A phase I clinical trial is currently underway studying dinaciclib and veliparib combination therapy (trial number NCT01434316, clinicaltrials.gov). Importantly, CDK12 deficiency was noted to be a significant biomarker for PARPi sensitivity in ovarian cancer cells, inducing synthetic lethality similar to *BRCA1/2* mutations [75].

PI3K-Akt-mTOR pathway inhibitors

The PI3K-Akt pathway is a cellular pathway that promotes survival and proliferation [76]. PI3K inhibitors in combination with PARPi treatment have been shown to have synergistic therapeutic effects in *BRCA1* deficient breast cancer and *BRCA1*-proficient triple negative breast cancer [77]. Another study found that PI3KCA-deficient ovarian cancer cells were more responsive to combination therapy with the PI3K inhibitor BKM120 and olaparib than monotherapy with either drug [78]. Akt can potentially be inhibited for cancer treatment, but no drugs have been FDA-approved at this time [79]. Inhibition of mTOR using everolimus is also an effective treatment option for several different cancer types, and has been approved for treatment in advanced breast cancer. Akt activates a signaling cascade that promotes the survival of the cell, and it could interfere with the ability of PARP-inhibitor to induce cellular death [80]. However, the role of Akt-inhibitors and mTOR-inhibitors in relationship with PARP-inhibitor resistance is not fully understood yet.

Molecular basis of drug resistance

Causes and mechanisms of drug resistance

Restoration of the BRCA1-PALB2-BRCA2 axis

Defective HR that occurs as a result of BRCA1, BRCA2, or PALB2 mutations is a key component for the overall efficacy of PARP inhibitors. The combination of PARP inhibition and loss of HR leads to cell death, and without one or the other, the cell will survive. Despite excellent clinical response to PARP inhibition, HR-deficient cells almost always become resistant by developing reversion mutations that either restore or bypass HR [81,82]. The mutations that cause HR-deficiency in *BRCA1/2* or *PALB2* genes are often frameshift mutations, leading to non-functioning proteins. With reversion mutations, the reading frame is restored and thus, so is some of the protein function. Since new mutations rarely occur at the original mutation sites, newly synthetized proteins are chimeric versions and contain deletions (Fig. 3). However, as critical functional domains are restored in the C-terminus, HR is no longer defective and cells become resistant to PARPi (Fig. 3). Acquisition of secondary mutations is a prominent mechanism of PARPi resistance, which has been observed in breast, ovarian, and pancreatic cancers. Such acquisition also confers resistance to platinum-derived agents, like cisplatin and carboplatin [83–85].

Demethylation of the promoter of the *BRCA1* gene, which is often hypermethylated in cancer and turned off, is another means to restore the expression of a fully functional BRCA1 [86] and thus reactivate HR. When HR is intact, RAD51 is recruited to sites of DNA damage to assist in the initiation of HR. This can be visualized by indirect immunofluorescence in cells [87,88] or immunohistochemistry (IHC) on tissue samples [89]. The restoration of HR in BRCA1/2 or PALB2 mutants is associated with detectable increases in RAD51 foci [90–92], suggesting that these foci may be used as screening markers for potential resistance to PARP inhibition or platinum therapies.

Reactivation of HR through expression of RAD51

In addition to the reversion mutations mentioned above, secondary somatic mutations in RAD51 paralogs have been identified and associated with PARPi resistance in BRCA1/2 mutated serous ovarian cancer cells [93]. These mutations, as well as mutations in RAD51 itself [94], are thought to restore RAD51's ability to mediate homologous sequence invasion in the homologous recombination process. This increase in HR cancels the synthetic lethal effects of PARPi treatment, and cells become resistant to the drug.

Similarly, downregulation of Early Mitotic Inhibitor 1 (EMI1) is associated with RAD51 stabilization and accumulation, subsequent restoration of HR and thus PARPi resistance. Mutations that cause downregulation of EMI1 are selected with a high frequency in triple-negative breast cancer order enabling them to evade Olaparib-induced cell death [95].

Interestingly, RAD51 is often found overexpressed in cancer and this is associated with poor prognosis [96–98], suggesting that high levels confer a selective advantage to tumor cells, possibly through drug resistance.

Mutations in NHEJ can compensate for lack of HR

Mutations in the components of the NHEJ pathway can lead to reactivation of HR in *BRCA1/2* mutant cells. In normal cells, BRCA1 favors HR directly by interacting with PALB2 and BRCA2 to promote strand exchange. However, it also prevents NHEJ by antagonizing 53BP1 [99]. 53BP1 is a key component of the NHEJ machinery, and plays an essential role in the DSB repair pathway choice [100–102]. It promotes NHEJ by limiting DNA end-resection, which is required for HR to occur. 53BP1 interacts with RIF1, REV7, and the Shieldin complex (SHLD1, SHLD2, SHLD3) and the resulting complex, referred to as the 53BP1-RIF1-REV7-Shieldin axis, inhibits resection [103–105]. Loss of any of the factors in this complex has been associated with PARPi resistance in BRCA1-deficient cells, but interestingly not in BRCA2-deficient cells [106]. It is thought that the inhibition of the 53BP1-RIF1-REV7-Shieldin axis allows for end resection then HR to occur in a BRCA1-independent and RNF168-dependent fashion. RNF168 is an E3 ubiquitin ligase that can bypass BRCA1 requirement through its direct interaction with PALB2 to initiate HR mechanism.

Another molecule that has been linked to PARPi resistance is Dynein Light Chain 1 (DYNLL1), which, like 53BP1, functions to inhibit DNA end resection in normal cells and thus favors NHEJ. While it has been suggested that DYNLL1 interacts with 53BP1 to inhibit end resection, or that DYNLL1 works by inhibiting components of the resection machinery such as MRE11 [107–109], the exact relationship between DYNLL1 and the 53BP1 axis is still unclear. Similar to the inactivation of 53BP1, loss of DYNLL1 is associated with PARPi-resistance [109].

Stabilization of the replication fork

Stalling of the replication fork is extremely dangerous for genome integrity, as unprotected forks that do not restart eventually collapse, leading to DSBs and loss of genetic information. In normal cells, BRCA2 and other DNA repair proteins contribute to fork protection [110,111]. In *BRCA1/2* mutants or cells exhibiting BRCA phenotypes in the absence of BRCA mutations (so-called "*BRCA-like*" cells), PARPi functionality leads to the accumulation of single stranded breaks that stall replication forks. PTIP and EZH2 then recruit the nucleases MRE11 and MUS81 to stalled forks, where they resect nascent strands and induce replication fork collapse (RFC) [110,112], resulting in double stranded breaks [57].

Stabilization of the replication fork through a variety of mechanisms has been seen as a potential mechanism of resistance to PARP-inhibitors. A frequent culprit is FANCD2, which is often expressed in BRCA1/2 mutant ovarian, breast, and uterine cancer cells. It also functions to protect the fork by suppressing MRE11-mediated fork collapse, and by stabilizing the fork, it leads to PARPi resistance [113]. Other proteins that promote resection of the fork in the absence of BRCA2, such as SMARCAL1, or replication forks remodelers such as HTLF and ZRANB3, also induce PARPi resistance when deleted [114,115]. SLFN11 is another important protein that has been linked to PARP-inhibitor resistance. SLFN11 functions to prolong S-phase of the replication cycle upon replication fork stress by inhibiting replication. This ultimately causes prolonged and irreversible stalling of the replication fork that is irreversible and can render a cell more susceptible to the effects of PARP-inhibitors [116]. Loss of SLFN11 can lead to resistance to PARPi-Temozolomide combination therapy [117].

Drug efflux pumps

A readily acquired mechanism of resistance to any class of drugs is the use of efflux pumps that transport drugs out of the cell, thus limiting their intracellular availability. *ABCB1* genes, also referred to as multidrug resistance (MDR1) genes, encode for p-glycoprotein efflux pumps that ultimately reduce the available amount of PARPi drug in the cell, leading to reduced efficacy of the drug and PARPi resistance, especially in *BRCA1*-deficient breast cancer and ovarian cancer cell lines [118,119]. Patients treated with chemotherapy prior to initiating PARPi therapy may be at increased risk for this resistance mechanism, as it has been demonstrated that the ABCB1 transporters are upregulated in non-naïve (*e.g.*, previously exposed to therapies) tumors as a result of chromosomal translocations that occur upon paclitaxel treatment [120]. MDR1 inhibitors, such as verapamil and elacridar, can reverse ABCB1-mediated drug-resistance in both olaparib- and paclitaxel-treated ovarian cancer cells [118]. Interestingly, paclitaxel-resistant ovarian cancer cell lines were also cross-resistant to olaparib, rucaparib, and doxorubicin but were not resistant to veliparib, which is not a substrate of p-glycoprotein efflux pumps. The above evidence highlights the difference between PARPi molecules, and the need for caution when prescribing olaparib and paclitaxel, with the upregulation of MDR1 being a significant risk factor in the resistance of cancer cells to treatment. Clinical trials involving MDR1 inhibitors have yielded poor outcomes, and recent studies point toward MDR1 being essential for an efficient immune

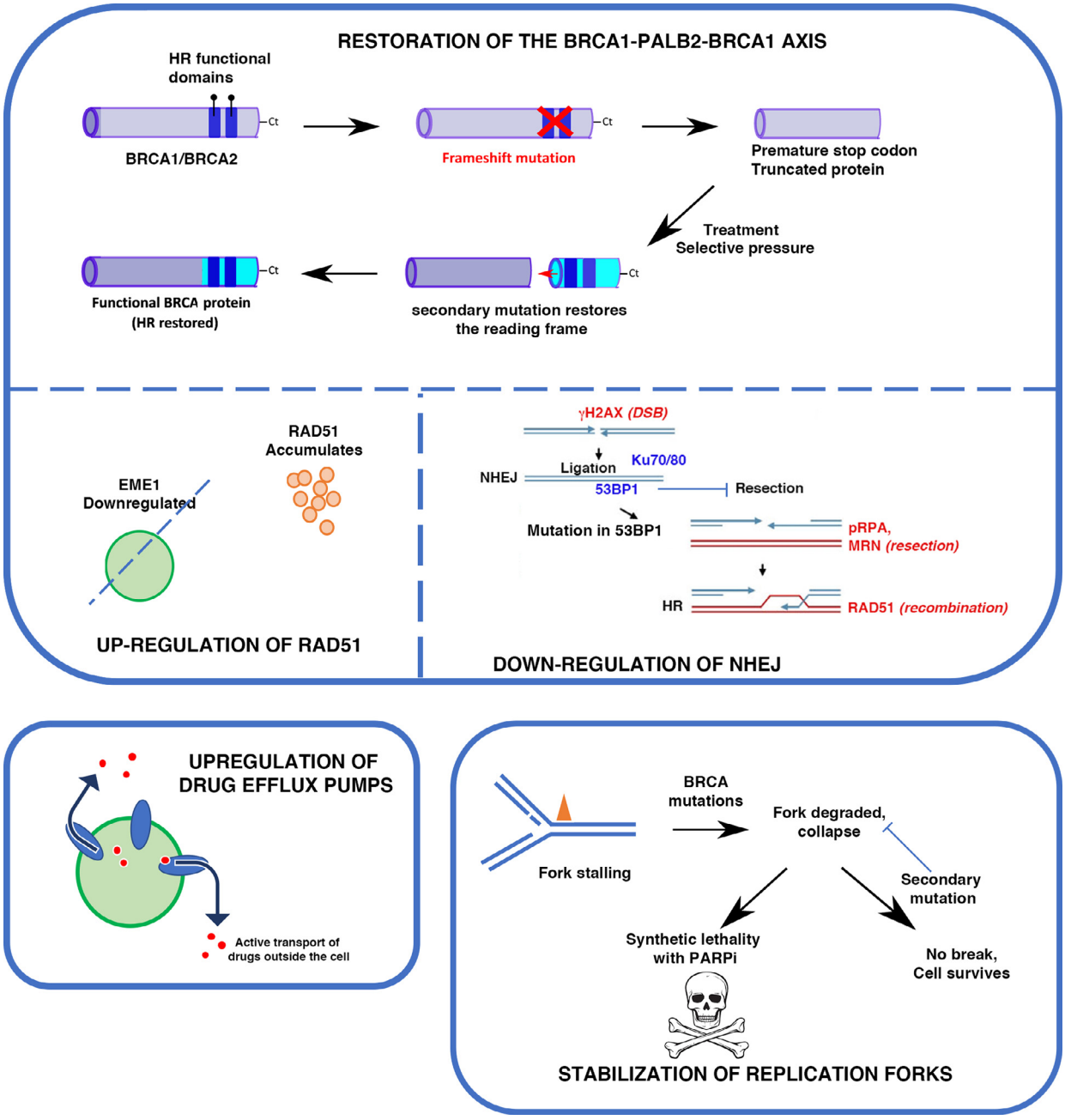

Fig. 3. Major mechanisms of resistance to PARPi. Top panel: Restoration of HR by acquisition of secondary mutations in HR genes, or in NHEJ genes. Bottom left panel: drug efflux pumps can decrease intracellular concentration of drugs such as PARPi. Bottom right: Stabilization of forks allow rescue even in the absence of other cellular components, and reverse the synthetic lethality, thus rendering cells resistant to PARPinhibition.

response [121]. While further studies are needed to delineate the role of MDR1 gene screening in designing treatment course, it remains very uncertain how effective regimens that combine low-dose MDR1 inhibitors with PARP inhibitors – to limit or reverse drug resistance – will be.

PARP-1 and PARG mutations

Mutations in the PARP1 molecule itself have been linked with the development of PARPi resistance, particularly by decreasing PARP trapping on damage DNA segments, the main mechanism by which PARPi work [122]. Interestingly, the specific types of BRCA1 mutations can tolerate mutations in PARP better than others. Mutations in exon-11 of BRCA1 leave the mutant BRCA1 protein partially functional, while frameshift mutations leading the deletion of C-terminal BRCT domains cause complete loss of BRCA1 function. Experimental evidence showed that the partially functional BRCA1 mutants were able to tolerate PARP mutations and survive, while cells with the frameshift mutated *BRCA1* genes were not able to survive with PARP mutations. Because of this, the partially functional mutant *BRCA1* genes with concomitant PARP1 mutations pose the risk of developing PARPi resistance through decreased PARP trapping. Frameshift BRCA1 mutations theoretically will not be resistant to PARPi through the mechanism of PARP mutation, because these cells would not be viable in the first place [122].

As briefly mentioned previously, PARG plays a role in removing PAR groups from multiple proteins in a process referred to as dePARylation. This process is necessary for the DNA repair process to occur. Loss of PARG in BRCA2-deficient cells that are being treated with a PARPi is thought to lead to partial restoration of PARylation so that there is decreased PARP trapping and also some return of DNA-damage repair sig-

naling, ultimately leading to PARPi resistance [123]. Studies conducted to assess mechanisms of resistance to PARPi in ovarian cancer cells detected reduced PARG levels in both HR-proficient and HR-deficient cell lines [124], supporting the notion that loss of PARG can contribute to the development of PARPi drug resistance. Interestingly, in cells that have not been previously treated with PARPi, inhibition of PARG appears to be synthetically lethal with HR deficiency [125], restoring PARPi sensitivity. It is postulated that PARG inhibition leads to dysregulation of PARylation and the accumulation of unrepaired DNA with subsequent replication fork stalling [126]. Further studies are warranted to define the relationship between PARG and PARP and what role PARG inhibition has in the treatment of HR-deficient or HR-proficient malignancies. In addition, TARG1 is a molecule that plays a similar role to PARG with respect to dePARylation and may be another potential target to prevent PARPi resistance, but further studies regarding this are warranted [127].

Therapeutic options for aquired drug resistance

Drug resistance is extremely common in advanced cancers. Overcoming this problem is therefore of paramount importance for improving treatment efficacy. With the success using PARP inhibition, yet the many potential mechanisms for PARPi resistance, there is an abundant body of research that looks to address interventions to restore PARPi sensitivity. Fortunately, a variety of pathways can be targeted and drugs under study show great potential for the reduction and even the reversal of PARP-inhibitor resistance.

ATR inhibitors

Three major kinases control the majority of the DNA damage responses in vertebrates, by acting both as sensors of the break and effectors of the repair signaling [128]. The kinases: ATM, ATR, and DNA-PK, are responsible for phosphorylating most DNA repair proteins, and by doing so activate proteins, allow protein-protein or protein-DNA interactions, and eventually DNA damage repair to occur. While mutations in their respective genes cause human disease, molecules have been developed against each of these kinases offer hope as combination therapy with genotoxic chemo-regimen. ATM deficiency has been shown to increase sensitivity to PARPi in lung adenocarcinoma, metastatic gastric cancer, metastatic prostate cancer, and lymphoid tumor cells, likely due to genomic instability and increased DNA damage in the absence of ATM's counteraction of toxic end-joining to allow the repair of DSBs [129–133]. This highlights the importance of screening for ATM-deficiency as a potential marker for PARPi-sensitivity. Additionally, combination of ATR-inhibitors with PARP-inhibitors in the treatment of ATM-deficient BRCA1/2 mutated tumors achieved therapeutic response at lower concentrations than monotherapy with either drug [134]. There may be a role for the combination of ATR-inhibitors and PARP-inhibitors in ATM-deficient cancer cells to reduce drug toxicities and resistance that is associated with prolonged use of high-dose drugs.

In addition to their treatment of ATM-deficient BRCA1/2 mutant cancer cells, ATR inhibitors in combination with PARP-inhibitors have been shown effective in the treatment of cancers with acquired *BRCA1/2* mutations and those with wild-type *BRCA1/2*. While ATM is the DNA repair upstream kinase, ATR's role is replication centric. Following DNA damages, ATR activates DNA replication checkpoints and rescues operations of stalled replication forks by signaling DNA damage and activating DNA repair proteins for their recruitment to damaged fork. ATR recruits PALB2 to sites of DNA-damage, both in the BRCA-dependent and BRCA-independent pathways [135]. Increased PALB2 recruitment to DNA damage sites restores HR and is linked to PARPi resistance [136,137]. Inhibition of ATR has the potential to reverse PARPi caused by secondary mutations in *BRCA1/2*, as it limits HR through PALB2 [138].

Combination treatment with ATRi and PARPi is a promising therapeutic avenue for drug resistant cancers. Currently, several clinical trials are underway to assess the role of AZD6738 (ceralasertib) in future cancer drug regimens (see [139] for a list of clinical interventions using this ATRi).

Cell cycle kinases inhibitors

WEE1 kinase functions to prolong the G2 phase of the cell cycle by phosphorylating CDK1, thus inhibiting it and preventing it from initiating progression of the cell cycle while cells repair DNA damages. One study showed that both HR-deficient and HR-proficient high-grade serous ovarian cancer cells with resistance to olaparib were sensitive to treatment with WEE1 kinase inhibitor, AZD1775 [124]. It is thought that WEE1 inhibitors promote more DNA damage and thus cell death in the absence of inhibitor CDK1 phosphorylation. Administration of a WEE1 inhibitor concurrently with a PARP inhibitor in ovarian cells is highly effective but toxic. Sequential therapy where PARPi are followed by administration of WEE1i is to be preferred as it is less toxic than the combination, and targets specifically cells with high replication stress thus killing preferentially tumor cells [140]. Clinical trials are currently underway to further assess the clinical role of AZD1775 in cancer treatment.

Another cell cycle kinase, CDK12 is thought to be involved in promoting the expression of DNA-damage response genes, such as *BRCA1, ATR, FAN1*, and *FANCD2*. Inhibition of CDK12 by dinaciclib has the potential to reverse primary and acquired PARP-inhibitor resistance in both BRCA-deficient and BRCA wild type triple-negative breast cancer cells, likely through increasing genomic instability [74]. A phase I clinical trial is currently underway studying dinaciclib and veliparib combination therapy. Moreover, CDK12 deficiency was noted to be a significant biomarker for PARPi sensitivity in ovarian cancer cells, inducing synthetic lethality similar to BRCA1/2 mutated cells when treated with a PARPi [75]. This highlights the importance of the use of genomic sequencing to better predict a specific cancer's likelihood of response to PARPi treatment. Although sequencing is not systematic in patient's management, the identification of specific signatures and the recent development of specific antibodies for immuno-histochemistry [141,142] have made it easier to use specific markers of drug response or drug resistance. Interestingly, deletion of CDK12 has been shown to reverse PARPi resistance regardless of the *BRCA* mutation status and is one of the most promising therapeutic options to counter PARPi resistance [73–75,143].

PARG inhibitors

BRCA2 mutation carrier cells that have defective HR demonstrate sensitivity to inhibition of PARG, a potential treatment avenue for HR-deficient cells. This effect from PARG-inhibition is not seen in cells with intact homologous recombination [144]. It is thought that PARG-inhibitors can induce accumulation of DNA damage and stalling of the replication fork by blocking the essential dePARYlation step that is required for efficient DNA repair [126]. To assess the synthetic lethality of PARG-inhibitors with homologous recombination deficiency, one study assessed the effects of PARG-inhibitors and PARP-inhibitors on PTEN-mutated glioblastoma multiforme cells. The study found that neither drug induced synthetic lethality in PTEN-mutated or PTEN-wildtype cells [126]. While PARG knock down is synthetic lethal with many HR genes, such as BRCA1, BRCA2, PALB2, FAM175A (ABRAXAS) and BARD1, studies have suggested that PARG inhibition *in vivo* might not be efficient to kill BRCA1-deficient tumors [145], and could even promote PARPi resistance. In the light of these conflicting data, and as new PARGi are being developed [146], further studies are required to uncover the potential role for PARG-inhibitors in cancer therapy and more specifically their role, if any, in PARPi resistance or reversion of it.

Direct inhibition of oncogene transcription

BRD4 promotes the transcription of several oncogenes by the recruitment of transcription factors or by binding to their enhancers. Inhibition of BRD4 causes deficiency of homologous recombination, resulting in synthetic lethality with PARP-inhibitor treatment. Interestingly, BRD4-inhibitors were found to reverse PARP-inhibitor resistance related to reversion of HR function, specifically through secondary BRCA1, RAD51C, and RAD51D mutations, loss of 53BP1, decreased PARP1 levels, and KRAS mutations. [147,148] Clinical trials assessing the safety of BRD4-inhibitors in combination with PARP-inhibitors are required for further evaluation of this treatment regimen in the role of clinical PARP-inhibitor resistance treatment.

Similar to BRD4-inhibitors, BET-inhibitors function by binding to and inhibiting bromodomains that are responsible for oncogenesis. BET-inhibitors have been found to inhibit BRCA1 and RAD51 expression, and have demonstrated synthetic lethality with PARP-inhibitors in the treatment of HR-proficient tumor xenografts [149,150]. Clinical trials are currently underway to study these drugs.

Conclusion

Whole genome sequencing and data mining using large datasets has contributed a better understanding of cancer etiology, the subclassification of patients into risk groups, and the establishment of genomic signatures. All this new information, when available, can help predict patient survival, response to drug treatment, and possibly even short- and long- term drug resistance.

While these advances in understanding specific predisposition to certain types of cancers for any given individual is powerful and can help design a therapeutic course, it has shown limitations. Not all mutations within the same gene yield similar cellular consequences, and researchers and clinicians alike are now trying to classify patients based on the clinicopathological features of their tumors rather than solely based on their inactivated genes. For example, while *BRCA1* and *BRCA2* genes are well-known tumor suppressors through their role in HR, their mutations can be mimicked by inactivation of other genes, and differentially impact on BRCA1/2 protein function. The shared clinical features and especially the sensitivity of such tumors for genotoxic agents (of physical and chemical) is defined as 'BRCAness'. The definition of BRCAness is a defect in HR repair, and high genetic instability that ensues, phenocopying deficiencies in *BRCA1* or *BRCA2*. As our understanding of BRCA1 and BRCA2 function grows, other roles, such as contribution to ubiquitination and replication fork protection respectively, can be included in BRCAness. Defining a "behavioral" pattern and predicting drug response for all tumors is the ultimate goal, and a major barrier in attaining patient- tailored, or personalized medicine for all.

Two major reasons that impair perfect prediction of long-term cancer behavior and drug response are (i) the complex biology of carcinogenesis (ii) the multiple mechanisms that can lead to drug resistance, and the difficulty to provide toxic prophylactic treatment in anticipation of resistances and recurrence. The development of combination therapies will hopefully identify additional candidates for tailored therapy by PARPi, ATRi, and other promising molecules.

Declaration of Competing Interest

All authors declared that there are no conflicts of interest.

Authors' contribution

All authors wrote the manuscript, E.D and P.D edited it, D.J prepared figures.

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