PARP inhibitor resistance in BRCA1/2 mutated tumors

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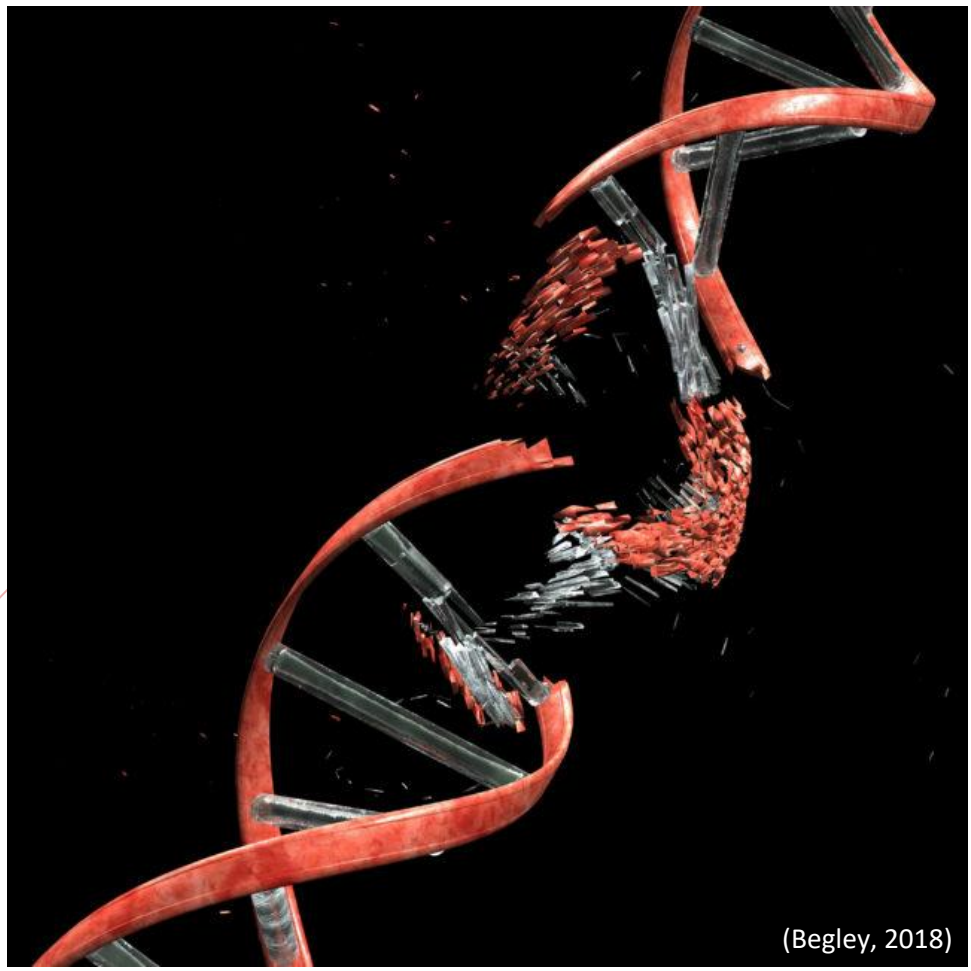

(Begley, 2018)

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Abstract

The most prevalent type of cancer in woman is breast cancer. Even though most types of breast cancers are curable, still 15% has triple negative breast cancer (TNBC) which is usually not curable. In addition, woman with breast cancer susceptibility protein (BRCA) mutations have up to 80% change of developing breast cancer and 40% chance of developing ovarian cancer. There is an unmet need for effective treatments of TNBC in particular but also ovarian cancer. A new treatment strategy is poly (ADP-Ribose) polymerase (PARP) inhibition. The mechanism of action of PARP inhibitors (PARPi) is based on synthetic lethality, meaning that these inhibitors only kill cells with two types of deficiencies. In the case of PARPi, homologous recombination (HR) deficient cells are killed. Since those cells are HR deficient a double stranded DNA (dsDNA) break can only be repaired with an error prone repair mechanism called non-homologous end joining (NHEJ). This can lead to mutations, deletions and chromosomal translocation which eventually can lead to cell death. Cells that are HR deficient rely on PARP to repair single strand DNA (ssDNA) breaks before they become dsDNA breaks, however when PARP is inhibited more dsDNA breaks will develop, resulting in cell death. And since HR is still effective in healthy cells, the healthy cells can repair the dsDNA damage despite PARP inhibition and will stay alive. PARP inhibition is not only useful in BRCA mutated types of cancer that lack HR DNA repair but also in cells that for any other reason are HR deficient. For instance because of epigenetic silencing of the promotor of the BRCA gene or because of BRCAness. Therefore PARPi seem to be a great step forward. However, there is also a problem considering PARPi, since resistance frequently develops against this drug. In this thesis, multiple PARPi resistance mechanisms will be described such as, upregulation of drug efflux pumps but also secondary mutations and poly (ADP-ribose) glycohydrolase (PARG) down regulation. In addition, possible solutions against the resistance will be mentioned.

Figure 1: Schematic view of the PARP protein in blue bound to DNA, in green the PARP inhibitor is shown (Victor, 2014).

List of abbreviations

Abbreviation	Name
53BP1	p53 binding protein 1
ABCb1a/b	ATP-binding cassette, sub-family B 1a/b
ALC	Chromodomain-helicase-DNA-binding protein 1-like
AML	Acute myeloid leukemia
ATM	Ataxia-telangiectasia mutated
BARD1	BRCA1-associated RING domain protein 1
BER	Base excision repair
BRCA	Breast cancer susceptibility protein
CDK1	Cyclin dependent kinase 1
CDK12	Cyclin dependent kinase 12
CHD4	Chromodomain-helicase-DNA-binding protein 4
DDR	DNA damage response
dsDNA	Double strand deoxyribonucleic acid
DYNLL1	Dynein light chain 1
ERK1/2	extracellular signal–regulated kinases 1/2
EZH2	Enhancer of zeste homolog 2
FANCD2	Fanconi anemia group D2
FDA	U.S. food and drug administration
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HGSOC	High-grade serous ovarian cancer
HR	Homologous recombination
HRD-LOH	Homologous recombination deficiency loss of heterozygosity
HSP90	Heat shock protein 90
MDS	Myelodysplastic syndrome
MiR-622	Micro RNA 622
MMEJ	Microhomology-mediated end joining
MMR	Mismatch repair
NAD^+	Nicotinamide adenine dinucleotide
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
ORR	Overall response rate
PALB2	Partner and localizer of BRCA2
PARG	Poly (ADP-ribose) glycohydrolase
PARP	Poly (ADP-ribose) polymerase
POLQ	DNA polymerase theta
PTEN	Phosphate and tensin homolog
shRNA	Short hairpin RNA
SIRT1	Sirtuin 1
SLFN11	Schlafen family member 11
ssDNA	Single strand deoxyribonucleic acid
TNBC	Triple negative breast cancer
TOPBP1	Topoisomerase 2-binding protein 1

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Introduction

Cancer is an uncontrolled growth of cells. This can either be caused by inherited genes or environmental exposure to carcinogens or simply by bad luck. Weinberg *et al*. wrote in their much citated work about the common hallmarks of cancer, such as chromosomal instability, sustaining proliferation signals and resisting cell death (Hanahan & Weinberg, 2011). In addition, defects in genome maintenance as well as repair pathways often occur in cancer. Sometimes specific DNA repair defects are seen which make the cancer cells rely on backup repair pathways to survive. Targeting these back-up mechanisms could be a new strategy for cancer therapy (Altmeyer, 2012). For instance when breast cancer susceptibility protein (BRCA) is mutated, for example in high grade serous ovarian cancer (HGSOC) or triple negative breast cancer (TNBC). HGSOC is also characterized by genomic instability (Parkes & Kennedy, 2016) and usually affects post-menopausal woman. It is the second most common malignant gynecological disease (Papa, Caruso, Strudel, Tomao, & Tomao, 2016). BRCA mutations have also been shown in TNBC, and more than 80% of the hereditary BRCA1 mutated cancers are actually TNBCs. TNBCs lack the expression of human epidermal growth factor receptor (HER2) as well as the estrogen and progesterone receptors (Dizdar, Arslan, & Altundag, 2015). Breast cancers consist of 15% TNBCs that are currently treated with platinum-based combination chemotherapy but there is no cure (Parkes & Kennedy, 2016). 50% of HGSOC and 10-20% of breast tumors, metastatic prostate cancers and pancreatic cancers have mutations in homologous recombination (HR) like for instance BRCA mutations (D'Andrea, 2018). In this thesis DNA repair and the function of BRCA will be explained. In addition, the reliance on PARP for the BRCA deficient cells will be shown. Also the function of PARP will be discussed during DNA repair in both healthy as well as BRCA deficient cells. In addition, PARP inhibitors (PARPi) will be discussed and how they could be used to treat specific cancers and how PARPi resistance could be treated.

DNA repair and BRCA

There are a lot of causes of DNA damage and when this is not sensed and repaired by the cell it can have serious consequences. Therefore, there are multiple pathways, divided in two groups, to repair DNA damage. The first group is involved in repair of single strand DNA (ssDNA) damage, which can be done by base excision repair (BER), mismatch repair (MMR) and nucleotide excision repair (NER). The other group repairs double strand DNA (dsDNA) damage, which is repaired by non-homologous end-joining (NHEJ), which is a fast process but causes a lot of errors or by HR, which is a slow process but generally doesn't cause mutations (see figure 2) (Papa, Caruso, Strudel, Tomao, & Tomao, 2016).

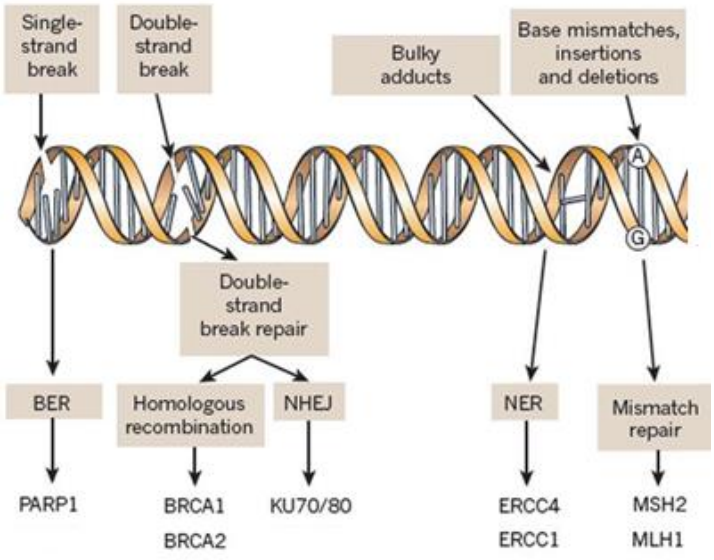

Figure 2: Overview of the different types of DNA damage and their corresponding DNA repair mechanisms. In addition, the proteins needed for the specific repair mechanisms are shown (Papa, Caruso, Strudel, Tomao, & Tomao, 2016).

HR is BRCA dependent (Dizdar, Arslan, & Altundag, 2015) and occurs during the S and G2 phase of the cell cycle (Turk & Wisinksi, 2018). ssDNA breaks can become dsDNA breaks when the break is not repaired and the replication fork is stalled. The replication fork can collapse due to platinum chemotherapy or ionizing irradiation. dsDNA damage is very toxic, when it is incorrectly or not at all repaired. This results in mutations, amplifications, chromosomal translocations and deletions which can result in senescence, cell death and cancer (Rabenau & Hofstatter, 2016; Noordermeer & Van Attikum, 2019).

BRCA stands for breast cancer susceptibility protein (GeneCards, 2019). There are two subtypes: BRCA1 located on chromosome 17 and BRCA2 located on chromosome 13 (see figure 3) (Turk & Wisinksi, 2018). Both are important for HR dsDNA repair. BRCA1 can interact with BRCA1-associated RING domain protein 1 (BARD1) to form a stable heterodimer which can bind to the damaged DNA (Dizdar, Arslan, & Altundag, 2015). In addition, BRCA1 can sense the DNA damage and link the DNA damage response (DDR) effectors (Roy, Chun, & Powell, 2016). Both BRCA1 as well as BRCA2 can bind to RAD51, which is a DNA recombinase, which then localizes to the damaged DNA (Lord & Ashworth, 2016). Both BRCA1 and BRCA2 can restart the stalled replication forks (Parkes & Kennedy, 2016). But it is thought that BRCA2 can also protect the replication fork by protecting the nascent DNA strand from degradation (Roy, Chun, & Powell, 2016).

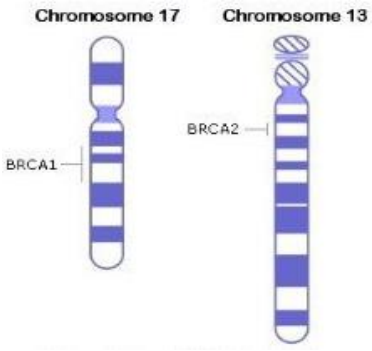

Figure 3: Location of the two BRCA genes (Anonymous, 2015).

In 33% of the ovarian tumors, deficient BRCA expression was seen by either somatic and germline mutations (20%) or by BRCA1 epigenetic silencing (11%) (Rabenau & Hofstatter, 2016). When BRCA1/2 is mutated, cells are HR deficient which can result in genomic instability (Dizdar, Arslan, & Altundag, 2015) and eventually cell death. When HR is deficient, dsDNA breaks are repaired by NHEJ which is error prone and introduces especially DNA deletions (Lord & Ashworth, 2016). 80% of the people with BRCA mutations do develop breast cancer. BRCA1 mutations usually result in earlier onset of cancer compared to BRCA2 mutations. In addition, in breast cancer BRCA1 mutation tumors are more likely to be TNBC tumors compared to BRCA2 mutation tumors (Turk & Wisinksi, 2018). BRCA mutations can also cause ovarian cancer which is seen in about 40% of the individuals with the mutation (MSK, 2019).

Sporadic breast cancers sometimes also have aberrant BRCA function or expression which is called BRCAness (Dizdar, Arslan, & Altundag, 2015). BRCAness can arise by somatic mutations but also by epigenetic silencing via hypermethylation of the promotor, which is reported in 31% of ovarian cancer cases and is the second most common HR aberration class (Parkes & Kennedy, 2016; Wakefield, Nesic, Kondrashova, & Scott, 2019; Kondrashova & Topp, 2018). Both BRCA1/2 mutation associated tumors as well as BRCA like tumors have a higher response rate to platinum-based chemotherapy (Parkes & Kennedy, 2016). HR deficiency can also be caused by other defects and gene mutations. In this thesis, most focus will be on TNBC and HGSOC with BRCA mutations since this has been investigated the most.

Poly(ADP-ribose) polymerase inhibitors

The poly (ADP-ribose) polymerase (PARP) protein family can catalyze the conversion of nicotinamide adenine dinucleotide (NAD^+) into long poly(ADP-ribose) (PAR) chains. PARP1 was the first protein described that can catalyze PAR formation, sense DNA damage and initiate the BER pathway to repair ssDNA damage. PARP1 consists of three functional domains: a catalytic domain, an auto modification domain and an amino-terminal DNA-binding domain (see figure 4) (Gagné, Rouleau, & Poirier, 2012).

In addition to initiating the BER pathway, PARP1 can also restart the stalled replication fork and induce NHEJ by binding to the damage site. HR-deficient cells are more dependent on NHEJ and therefore rely on PARP1 (Kaufmann, 2010; Dizdar, Arslan, & Altundag, 2015; Chassé, et al., 2017; Altmeyer, Messner, Hassa, Fey, & Hottiger, 2009).

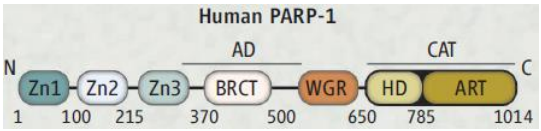

Figure 4: Structure of human PARP1 with the different domains. Shown the Zinc fingers, AD, WGR and CAT (Gagné, Rouleau, & Poirier, 2012).

PARP1 can also facilitate repair by sensing unligated Ozaki fragments during DNA replication (Francica & Rottenberg, 2018). Research showed that p53-/- mice with a disrupted PARP1 gene have a high incidence of tumors (Tong, et al., 2003). When there is DNA damage, PARP1 is localized to the site of DNA damage. PARP1 causes a post-translational modification by adding an ADP-ribose to the target acceptor protein (usually a glutamate or aspartate) with an ester bond. Some PARPs can repeat adding ADP-ribose units with a ribose-ribose bond (PARylation) (Turk & Wisinksi, 2018; Maudry, et al., 2016; Tallis, Morra, & Barkauskaite, 2014). This results in relaxation of the chromatin because of the big negative charge from the polymers (Sellou, et al., 2016). Relaxation of the chromatin, mediated by chromodomain-helicase-DNA-binding protein 1-like (ALC1), is important for the accessibility of specific repair proteins but also transcription factors (Marjanovic, Crawford, & Ahel, 2017; Kim, et al., 2017). When there is too much or too little ALC1, the cells become more sensitive to DNA damaging agents (Ahel, et al., 2009). Relaxation is followed by recruitment of DNA repair proteins to the site of damage (Bryant, et al., 2005) which leads to DNA damage repair (see figure 5). PARG erases PAR, which levels need to be low for replication fork progression (Wakefield, Nesic, Kondrashova, & Scott, 2019). PARP1 -/- mice are viable and don't show early onset tumors. However, the replication forks in those mice do collapse because of ssDNA breaks and this triggers HR (Bryant, et al., 2005).

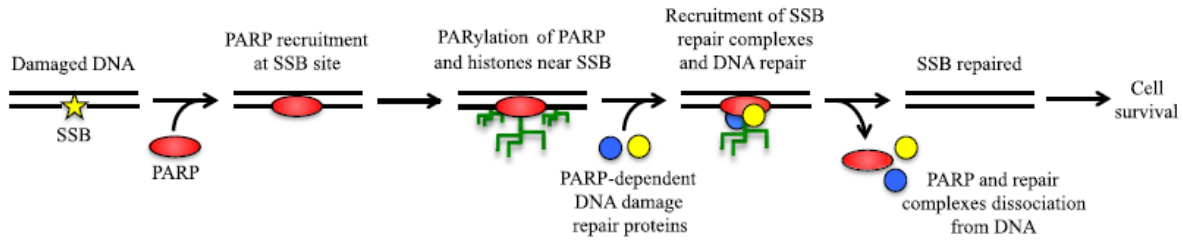

Figure 5: PARP mediated DNA damage repair mechanism in healthy cells. PARP is localized to the damaged DNA. Then PARylation is started resulting in open chromatins which can recruit DNA repair proteins. The DNA damage is then repaired and the cell survives (Livraghi & Garber, 2015).

Transcriptional activity is modulated by PARP by affecting SIRT1 since PARP can deplete the NAD^+ pool which is necessary for sirtuin 1 (SIRT1). SIRT1 can acetylate genes and it can also negatively regulate PARP1 via deacetylation and regulation of PARP1 transcription (Marjanovic, Crawford, & Ahel, 2017). In addition, SIRT1 can block PARP1 induced cell death (Piao, Fujioka, Nakakido, & Hamamoto, 2018). Reversal of PARP signaling is important since depletion of cellular NAD^+ leads to necrosis and too much PAR can cause apoptosis. PAR is mostly removed by poly (ADP-ribose) glycohydrolase (PARG), that cleaves the special ribose-ribose bindings, resulting in free ADP-ribose (Tallis, Morra, & Barkauskaite, 2014). PARP1 can be activated by extracellular signal–regulated kinases 1/2 (ERK1/2). In addition, methylated PARP1 shows to be more active compared to unmethylated PARP1. PARP1 can also be acetylated by p300 which is important for the interaction with p50. P50 is a component of NF-kB which is involved in inflammatory responses (Piao, Fujioka, Nakakido, & Hamamoto, 2018).

PARP activation is also important in the pathogenesis of some inflammatory and cardiovascular diseases. During apoptosis PARP1 is cleaved in two fragments by caspases. They recognize DEVD motif and cleavage separates the DNA binding domain from the catalytic domain which makes the enzyme inactive. The cleavage fragments suppress PARP activity. This feedback loop suggests that PARP1 activation prevents apoptosis. However apoptosis is needed to get rid of cells that are unfunctional in damaged organs. Therefore inhibition of PARP could improve the function of the damaged organs (Graziani & Szabo, 2005).

PARP1 is also important for epigenetics. PARP1 is a genome-wide memory mark for epigenetics in mitotic chromatin and it's important for the reactivation of transcription after mitosis. When PARP1 is activated, PAR is assembled. PAR affects the dimerization of PARP1 which leads to dissociation and loss of activity. This limits the time during which PARP1 remains active. PAR is negatively charged which can participate in electro-repulsive shuttling of chromatin proteins. This results in chromatin loosening and improved accessibility of the DNA for transcription factors and the general transcription machinery, showing that PARP can function as a chromatin architect (Lodhi, Kossenkov, & Tulin, 2014).

In 2005, the sensitivity of BRCA mutated cells to PARP inhibition was first shown (Bryant, Schultz, Thomas, Parker, & Flower, 2005; Farmer, McCabe, Lord, Tutt, & Johnson, 2005). Inhibition of PARP works via the synthetical lethality principle. This means that individual mutations are not lethal, however when they coexist, cells will die (Rabenau & Hofstatter, 2016; Dizdar, Arslan, & Altundag, 2015). In the case of PARP inhibition, the BER pathway will be compromised by competing with NAD^+ resulting in reversible inhibition. The ssDNA breaks will encounter the DNA replication fork and become dsDNA breaks repaired by HR. However, in HR deficient cells it will be repaired with NHEJ, resulting in cell death (see figure 6) (Dizdar, Arslan, & Altundag, 2015; Francica & Rottenberg, 2018).

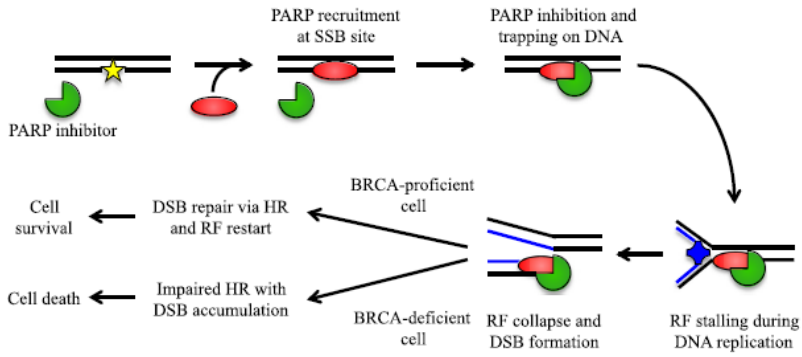

Figure 6: PARPi pathway in healthy and BRCA deficient cells. PARP is recruited to the ssDNA damage trying to PARylate the chromatin. However, the inhibitor binds to PARP resulting in a stalled replication fork which leads to dsDNA damage. In healthy cells dsDNA damage in the form of HR will be started and the cells will survive. However, in HR deficient cells NHEJ will 'repair' the dsDNA leading to a lot of mutations and eventually cell death (Livraghi & Garber, 2015).

In addition to synthetical lethality (see figure 7), PARP trapping is proposed as a mechanism of cell death in HR deficient cells (Dizdar, Arslan, & Altundag, 2015). Because of the PARPi, PARP can't be dissociated from the DNA breaks anymore which prevents proteins responsible for repair to access the DNA damage site (Dizdar, Arslan, & Altundag, 2015; Rabenau & Hofstatter, 2016) which prevents BER from happening (Parkes & Kennedy, 2016). When a PARPi can trap more PARP-DNA complexes, it is expected that it will cause less resistance against this PARPi (Parkes & Kennedy, 2016; D'Andrea, 2018).

Recently a third mode of action has been found for PARPi. Researchers showed that HR deficient cells rely on microhomology-mediated end joining (MMEJ) for dsDNA repair which uses a small homologous sequence for DNA repair. This pathway leads to deletions and needs both PARP1 as well as DNA polymerase theta (POLQ). PARP1 is necessary to localize POLQ to the dsDNA break which catalyzes DNA synthesis. And therefore PARP1 or POLQ inhibitors will inhibit MMEJ and actually kill the HR deficient cells (D'Andrea, 2018). Currently POLQ inhibitors are being developed (Pomerantz, 2018).

Researches showed that PARPi are beneficial for patients with BRCA mutations, however, not only BRCA patients respond. In addition, patients with RAD51 mutations and BRCA silencing respond as well (Wakefield, Nesic, Kondrashova, & Scott, 2019; Dizdar, Arslan, & Altundag, 2015). Furthermore, Phosphatase and tensin homolog (PTEN) deficiency can also cause HR defects in human tumor cells and therefore, PARPi are also potentially useful in PTEN deficient cells (SR, 2009). A better efficacy of PARPi is seen in HGSOC compared to breast cancer, which could be explained by lower BRCA1/2 mutation rate in breast cancer or because of biologic heterogeneity (Dizdar, Arslan, & Altundag, 2015).

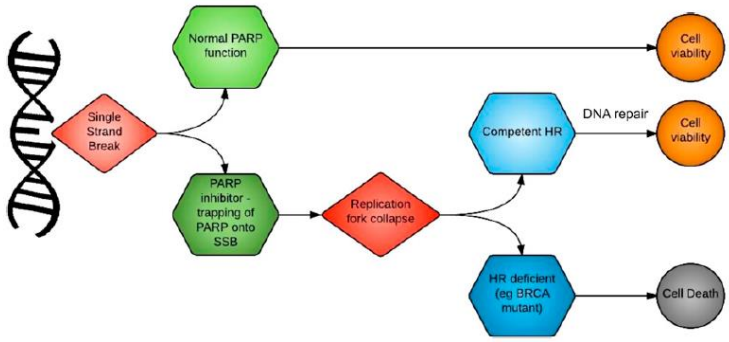

Figure 7: Synthetic lethality principle for BRCA deficient tumor cells. ssDNA damage in cells with normal PARP function is no problem. However when PARP is inhibited it will result in collapse of the replication fork which again is no problem when HR is still working well. However, when in addition to the inhibition of PARP also HR is deficient, cells will die (Parkes & Kennedy, 2016)

PARPi can be used as single agents since tumor cells duplicate often and with that have more DNA damage compared to healthy cells. Therefore they relying more on DNA damage reparation mechanisms. An article of Papa *et al*. showed 30-45% overall response rate (ORR) with PARPi used as single agent (Papa, Caruso, Strudel, Tomao, & Tomao, 2016) and a study of Livraghi *et al*. showed a slightly higher success rates of 33-65% (Livraghi & Garber, 2015). PARPi can also be used in combination with other therapies that induce more DNA damage and thus more cell death. However, when the cells become resistant, it is both to PARPi as well as the co administered drugs. In addition, more side effects are seen. PARPi can also be used as a maintenance therapy (Papa, Caruso, Strudel, Tomao, & Tomao, 2016; Livraghi & Garber, 2015). And since the chance of developing breast or ovarian cancer is 90% in individuals that have BRCA mutations, PARPi could also potentially be used as prevention to kill of all HR deficient cells (Dizdar, Arslan, & Altundag, 2015). Different PARP inhibitors have been made. They have different structures and implications, however they have the same working mechanism.

Olaparib was in 2014 the first PARPi approved by the U.S. food and drug administration (FDA). It was first trialed in patients with various cancers and BRCA1/2 mutations (Dizdar, Arslan, & Altundag, 2015). However, it is approved as a monotherapy for germline BRCA mutation patients with advanced ovarian, fallopian tube or primary peritoneal cancers. Patients that have platinum sensitive tumors seem to be the most sensitive for olaparib as well. As maintenance therapy it increases the progression free survival with most effect in patients with BRCA mutations sensitivity for platinum treatment. Later the FDA approved it for this purpose as well (Rabenau & Hofstatter, 2016). Trials also showed the benefit of olaparib in BRCA mutated prostate cancer patients as well as familial pancreatic cancer patients with BRCAness, which accounts for 19% of those patients. In addition, 5% of the gastric cancers do have a BRCA mutation. Recent phase II trials showed that these patients benefit from olaparib as a maintenance treatment (Rabenau & Hofstatter, 2016).

Currently, niraparib is the only PARPi that is approved by the FDA as a maintenance treatment for woman with ovarian cancer, independent of their BRCA status. It was approved in 2017 and is used as a maintenance treatment for ovarian cancer patients who did respond to platinum-based therapy in the past (Weintraub, 2017).

Rucaparib was approved in 2016 for BRCA mutated ovarian cancer (Bankhead, 2016) and talazoparib in 2018 for BRCA mutated breast cancer (Jarvis, 2019).

Since DNA repair is inhibited, PARPi could result in carcinogenesis, in particular myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). However the incidence of new cancers, seems to be lower than 1%. Noteworthy, the first approved drug is only 5 years old, so long term survival rates and follow ups are not known yet. In addition, patients treated with PARPi were initially treated with DNA damaging chemotherapeutics, which are known to increase the risks of AML as well (Dizdar, Arslan, & Altundag, 2015; Livraghi & Garber, 2015; Rabenau & Hofstatter, 2016). In addition, a few side effects have been found in small numbers. The main dose limiting toxicity of PARPi seems to be thrombocytopenia (decrease of platelets number in the blood) (Mason, 2010). Furthermore myelosuppression (decrease in red blood cells) was seen in some patients which is probably caused by sensitization of the bone marrow to chemotherapeutic agents by the PARPi (Parkes & Kennedy, 2016).

PARP inhibitor resistance

PARPi resistance is a big issue in the treatment of BRCA mutated tumors. Multiple mechanisms of PARPi resistance have been found in mice. It has been shown that long-term treatment with olaparib results in drug resistance caused by an up-regulation of the ATP-binding cassette, sub-family B 1a/b (ABCb1a/b) genes which encode for P-glycoprotein efflux pumps. Increased expression of these efflux pumps results in more efflux of the medicine which is seen in tumor cells but also BRCA1 mutated breast cancer mice models (Rottenberg, et al., 2008; Parkes & Kennedy, 2016; D'Andrea, 2018). This type of required resistance is often seen related to chemotherapeutic resistance (Wakefield, Nesic, Kondrashova, & Scott, 2019). It can be reversed by administration of tariquidar which is an P-glycoprotein inhibitor. It is also predicted that P-glycoprotein expression is directly modulated by PARP1 inhibition since PARP-/- mice have increased P-glycoprotein expression (Rottenberg, et al., 2008; Dizdar, Arslan, & Altundag, 2015). Upregulation of P-glycoprotein drug efflux pumps was also seen in colon cancer cells resulting in resistance to KU-58948, a PARPi from axon MedChem. This was restored by administration of verapamil which inhibits P-glycoprotein (Altmeyer, 2012).

Another mechanism of resistance that is often seen is genetic reversion of BRCA1/2 mutations as a result of secondary mutations in, for instance, p53 binding protein 1 (53BP1) (Dizdar, Arslan, & Altundag, 2015; Francica & Rottenberg, 2018). Loss of 53BP1, a genome caretaker, can restore HR in BRCA1-deleted cells. 53BP1 maintains the balance between NHEJ & HR. When short hairpin RNAs (shRNA) were used against 53BP1 in BRCA1-defective breast cancer cells, the PARP inhibition was reduced (Altmeyer, 2012; Francica & Rottenberg, 2018). Loss of proteins that are involved in NHEJ, like REV7 and RIF1, can also restore the HR function. They are downstream factors of DNA repair mediated by 53BP1, and similar to the loss of 53BP1, loss of REV7 or RIF1 results in a shift towards HR. When BRCA1 mutant protein is partially functional, it can gain full function by inhibition of heat shock protein 90 (HSP90) (Parkes & Kennedy, 2016; Francica & Rottenberg, 2018). The shieldin complex also functions downstream of the 53BP1 pathway. It localizes to the dsDNA break, this impairs NHEJ which then leads to the shift towards HR and results in PARPi resistance in BRCA1 deficient cells. It is unknown why this doesn't lead to resistance in BRCA2 deficient cells (Francica & Rottenberg, 2018). Dynein light chain 1 (DYNLL1) can also bind to 53BP1, which stimulates the recruitment of DYNLL1 bound to 53BP1 to dsDNA damage sites. In addition, it also stabilizes the interaction with DNA damage associated chromatin (Francica & Rottenberg, 2018). It is also possible that the second mutation causes a frameshift resulting in restoration of the open reading frame and expression of a nearly full length HR protein. This mechanism is usually seen later in the disease progression and results in a highly sub clonal phenotype of the tumor (Wakefield, Nesic, Kondrashova, & Scott, 2019; D'Andrea, 2018). HR function can also be restored by demethylation of the BRCA1 promotor. However, this could lead to heterogenous tumors (D'Andrea, 2018) and thus potentially cause partial PARPi resistance which is not ideal.

Cyclin-dependent kinase 12 (CDK12), a transcriptional regulator of HR, can also cause PARPi resistance by restoration of HR. In addition, some BRCA mutated cancers can display de novo resistance by hypomorphic isoforms of BRCA1 (Johnson, et al., 2016).

PARG levels are also important for PARPi resistance. When there is too much polymerized PAR, for instance because of downregulation of PARG, PARP1 can bind less to the DNA damage site which leads to a reduced effect of PARPi (Wakefield, Nesic, Kondrashova, & Scott, 2019). Francica *et al*. showed that endogenous PARG activity is needed for successful PARPi therapy. When there is no PARG then PARP1 can still PARylate even when the inhibitors are used (Francica & Rottenberg, 2018). In addition, when PARG is downregulated, PARPi effects are decreased, because PARG restores the recruitment of downstream factors for DNA damage repair. Shlafen family member 11 (SLFN11) is also important for the PARPi sensitivity. When cells do have SLFN11 they go in irreversible G1/S cell cycle arrest after PARPi treatment, which is lethal. However, when SLFN11 is depleted cells reenter the cell cycle and cells become less sensitive to PARPi (Gogola, Rottenberg, & Jonkers, 2019).

Replication fork protection failure is very important aspect of the PARPi synthetic lethality mechanism, and therefore it also plays an important role in resistance to PARPi. Usually BRCA can protect nascent DNA at replication forks that are stalled. In normal cells, those forks are protected by RAD51 against MRE11 which increases the stability of the fork. Protection of the stalled replication fork can be restored by numerus ways and thus lead to PARPi resistance. For instance, loss of proteins which are important for the remodeling of the replication forks or MRE11 chromatin modify complex but also by enhanced action of RAD51 or loss of chromodomain-helicase-DNA-binding protein 4 (CHD4) and enhancer of zeste homolog 2 (EZH2). In particular downregulation of EZH2 leads to downregulated MUS81 recruitment to the replication fork. Upregulation of Fanconi anemia group D2 (FANCD2) can induce PARPi resistance (Wakefield, Nesic, Kondrashova, & Scott, 2019; D'Andrea, 2018).

It is also absolutely necessary for PARP1 to be able to bind to DNA in order for the PARPi to work. So when the zinc fingers are mutated and not able to bind to the DNA anymore, PARPi resistance is seen (Wakefield, Nesic, Kondrashova, & Scott, 2019). And since the function of PARPi is to block the enzymatic action of PARP enzymes, PARPi resistance could also be sustained by a decrease in PARP enzyme expression (D'Andrea, 2018).

Overexpression and downregulation of microRNAs (small 22 nucleotide long non coding RNAs that inhibit translation of mRNA) can also induce PARPi resistance. For instance overexpression of micro RNA 622 (miR-622) decreases 53BP1, Ku70 and Ku80 which leads to PARPi resistance (Kim, et al., 2017).

Finally, DNA topoisomerase 2-binding protein 1 (TOPBP1) is also important in the sensitivity of PARPi. TOPBP1 is important in HR, it regulates the phosphorylation of RAD51 which is a downstream protein of HR. When TOPBP1 is lost, the cells become more sensitive for PARPi (Maudry, et al., 2016).

Not all tumors with BRCA mutations do respond to PARPi. In addition, some tumors without BRCA mutations actually do. Therefore, it is very important to be able to predict which tumors will respond and which won't also in consideration of the resistance problem. Biomarkers could be useful for this.

PAR levels could potentially be used as a candidate biomarker. However, technically it is difficult to detect PAR in tissue biopsies. So further research is needed to see whether this is feasible. Rad51 loading could also be used. However some HR defects don't show reduced Rad51 levels and sometimes synthetic lethality is even caused by mechanisms that are not dependent on HR. Tumors with mutations in other DNA repair genes like Rad53, Ataxia-telangiectasia mutated (ATM) gene, partner and localizer of BRCA2 (PALB2) and CHEK2 may benefit from PARPi as well (Rabenau & Hofstatter, 2016). Therefore a single biomarker could be misleading (Altmeyer, 2012).

There are also assays that can detect HR deficiency like homologous recombination deficiency loss of heterozygosity (HRD-LOH) assay, Myriad's HRD assay and measuring the genomic scar. Those assays however do not take in account that reverse mutations can induce HR again. This happens in 28% of the patients. Therefore identification of a pattern of gene expressions might be more beneficial (Dizdar, Arslan, & Altundag, 2015; Parkes & Kennedy, 2016).

Since PARG is necessary for PARPi efficacy (Francica & Rottenberg, 2018), this protein should also be taken in account when looking whether a patient is suitable for PARPi. However, this should not be the only biomarker. In addition, epigenetic silencing of both BRCA1 alleles is necessary for full PARPi effect, because when only 1 allele is silenced, resistance occurs (Francica & Rottenberg, 2018). Turk *et al.* wrote about a 77 gene signature which would be able to predict the BRCAness and thus the potential effectiveness of PARPi (Turk & Wisinksi, 2018). However this needs to be researched more.

How to prevent PARP inhibitor resistance

When HR is deficient as seen, for instance, in BRCA mutated tumors, the genomic instability increases due to the more error prone NHEJ mechanism used to repair DNA damage. This increased genomic instability could be associated with an elevated neo-antigen load which has been associated with a stronger anti-tumor immune response (Turk & Wisinksi, 2018). Meaning that those types of tumors with a HR deficiency and a high genomic instability could potentially be treated with immune therapy. Immune therapy has been tested in BRCA mutated tumors, for instance in ovarian cancers where the first case studies seem promising (Matsuo, et al., 2018).

We could also induce HR deficiency to treat more patients with PARPi as shown in the article of Kamel *et al*. One way to do this is by using a PI3K inhibitor called BKM120. This drug downregulates the activity of BRCA1/2 resulting in HR deficiency (Kamel, Gray, Walia, & Kumar, 2018). For this purpose, Cyclin-dependent kinase 1 (CDK1) inhibitors, CDK12 inhibitors, histone deacetylase (HDAC) inhibitors and HSP90 inhibitors could also be used (D'Andrea, 2018). In addition, using HR inhibitors to maintain the HR deficiency in tumors should also be considered. This could be done using dinanciclib which has the same results as a CDK12 knockout in mice. In addition, it was even shown BRCA mutated TNBC cells with acquired PARPi resistance can be resensitized to PARPi by dinanciclib (Johnson, et al., 2016). HR deficiency could also be induced via epigenetic silencing. When the BRCA gene is methylated it is silenced, this could manually be induced. If it is possible to specifically induce BRCA methylation in cancer cells this could be a reversible way to induce HR deficiency with potentially less risks.

Hyperthermia also could be used to prevent PARPi resistance. Hyperthermia results in membrane damage, protein denaturation and inhibition of both DNA repair as well as cell proliferation. Hypothermia in combination with PARPi will kill cancer cells because of synthetic lethality. Thermotolerance can be developed but this can be controlled with HSP90 inhibitors. Moreover, researchers found that combination of olaparib, HSP90 inhibitors and mild hyperthermia resulted in complete loss of tumor growth in xenograft mice models (Kim, et al., 2017). Noteworthy, because of the variety of functions of HSP90, toxicity needs to be kept in mind (Neckers & Workman, 2012).

Addition of HDAC inhibitors, increases sensitivity of cancer cells for PARPi according to BRCAness. This is especially the case in TNBC. The HDAC inhibitors block deacetylation which leads to hyperacetylation and inhibition of HSP90. Therefore BRCA cannot interact with HSP90 anymore which leads to degradation of the BRCA protein. Enhancing BRCA export to the cytoplasm can also be an interesting pathway to reduce the PARPi resistance, since BRCA can't execute its DNA reparation function anymore. This could be done by inhibiting BARD1 which is needed for the transport of BRCA1 to the nucleus (Kim, et al., 2017). To the best of my knowledge, no BARD1 inhibitors have been produced yet.

Conclusion

In the fight against cancer, in particular cancers with HR deficiency like BRCA mutated TNBC and HGSOC, new treatments options are needed and ultimately cures are hoped to be found. New drugs with a lot of potential for this subgroup of tumors are PARPi, which work on a synthetic lethality principle and specifically kill HR deficient cells. However, resistance is a major issue in this treatment, caused by multiple mechanisms. A well known resistance mechanism is the increase of P-glycoprotein efflux pumps because of up-regulation of the ABCb1a/b gene expression. The most common mechanism, however, is the genetic reactivation of BRCA mutations either by additional mutations or by epigenetic reversion resulting in functioning HR. Loss of PARG can also cause resistance and PARP1 function is also needed for PARPi to work (see figure 8).

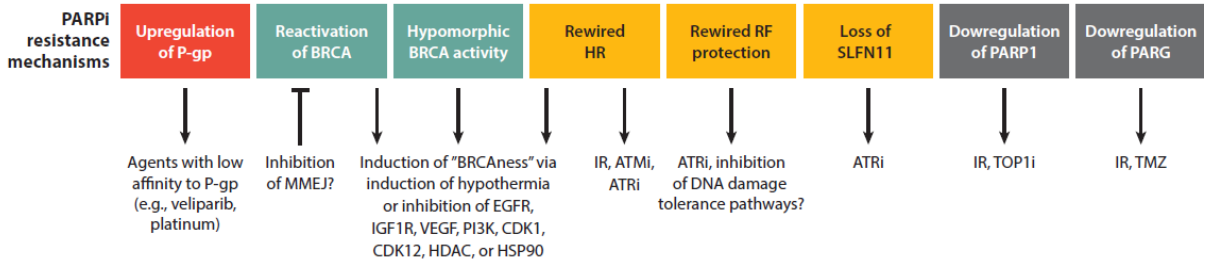

Figure 8: Different PARPi resistance mechanisms and their treatments that can resensitized the cells again (Gogola, Rottenberg, & Jonkers, 2019).

Even though all those mechanisms of resistance, drugs are available to treat resistance and to prevent it from happening (see figure 8). For instance, veliparib can be used to prevent upregulation of P-glycoprotein efflux pumps and ionizing radiation can be used to prevent downregulation of PARP1 and PARG. To prevent PARPi resistance from happening all drugs could be given at the start of the PARPi treatment. However, this will not be feasible regarding the side effects and comfort of the patients. In addition it will be very expensive. Therefore the best option would be to wait and see whether PARPi resistance start to develop. Then it is necessary to know which mechanism causes the resistance to give the proper treatment to re-induce PARPi effect. In addition, it would be nice to have good biomarkers to predict whether it is worth treating a patient with PARPi. And it would also be nice if we would be able to predict whether a patient will become resistant to PARPi and possibly even which mechanism will be most likely to treat them in advance with the drugs to prevent them from becoming resistant. The best solution would be too prevent PARPi resistance from occurring at all. However, since there are so many mechanisms that can cause PARPi resistance this is hard. Since increased efflux and reactivation of BRCA are the two mechanisms that are the most common to occur I would put my money on this in the fight to prevent PARPi resistance to develop. When we have PARPi resistance in control we potentially even could look at inducing HR deficiency in more tumors to be able to target them with PARPi. However, the challenge will be making specifically the tumor cells HR deficient and not the healthy cells to prevent serious side effects. Overall, with more research PARPi look promising to become standard therapy for more HR deficient tumors and potentially in the future for all tumors.

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