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REVIEW

Inhibiting the DNA damage response as a therapeutic manoeuvre in cancer

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The DNA damage response (DDR), consisting of an orchestrated network of proteins effecting repair and signalling to cell cycle arrest, to allow time to repair, is essential for cell viability and to prevent DNA damage being passed on to daughter cells. The DDR is dysregulated in cancer with some pathways up-regulated and others down-regulated or lost. Up-regulated pathways can confer resistance to anti-cancer DNA damaging agents. Therefore, inhibitors of key components of these pathways have the potential to prevent this therapeutic resistance. Conversely, defects in a particular DDR pathway may lead to dependence on a complementary pathway. Inhibition of this complementary pathway may result in tumour-specific cell killing. Thus, inhibitors of the DDR have the potential to increase the efficacy of DNA damaging chemotherapy and radiotherapy and have single-agent activity against tumours with a specific DDR defect. This review describes the compounds that have been designed to inhibit specific DDR targets and summarizes the pre-clinical and clinical evaluation of these inhibitors of DNA damage signalling and repair.

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Introduction and background

The integrity of the genome is essential for viability, and it is by damaging DNA that radiotherapy and most conventional anti-cancer chemotherapy exert their anti-cancer effect. DNA repair mechanisms can compromise the anti-cancer activity of these agents. Such mechanisms, of course, did not arise merely to thwart the attempts of clinicians to cure cancer; they evolved to cope with the daily onslaught of endogenous and environmentally induced DNA damage that every living organism faces. Thus, millions of years of evolution have given us a sophisticated battery of mechanisms to deal with the different types of DNA damage that we encounter on a continuous basis. The mechanisms that make up the complex DNA damage response (DDR) include not only the repair pathways but also the activation of cell cycle checkpoints that arrest the cell to allow DNA damage to be repaired either before it is fixed by replication or transmitted to the daughter cells at mitosis (Figure 1). The major DNA repair pathways are base excision repair/single-strand break repair (BER/SSBR), nucleotide excision repair (NER), DNA mismatch repair (MMR), non-homologous end-joining (NHEJ) and homologous recombination repair (HRR). BER/SSBR and NER deal with lesions affecting one strand of the DNA; MMR deals with replication errors (base mismatches, insertions and deletions); and NHEJ and HRR deal with DNA double-strand breaks (DSBs). DNA cross-links are dealt with using components of NER and HRR, along with the Fanconi's anaemia proteins. ATM and ATR are kinases that recognize DNA DSB and set off a cascade of phosphorylation reactions that promote repair and activate cell cycle arrest via CHK2 and CHK1.

Dysregulation of the DDR leads to the genomic instability that is an enabling characteristic for cancer development (Hanahan and Weinberg, 2011). DDR genes can therefore be considered as a subset of tumour suppressor genes, and defects in DNA repair and damage signalling are common in cancer. Defects in the p53/pRb pathway, which signal DNA damage to the G1 checkpoint, are probably the most common, but there are a number of syndromes associated with DNA repair defects. These repair defects include Lynch syndrome, which causes hereditary non-polyposis colorectal cancer, gastric, endometrial and ovarian cancer, and is due to mutations in MMR genes (Lynch and de la Chapelle, 1999; Barrow *et al.*, 2008); Fanconi's anaemia, which is associated with a high incidence of haematological malignancies, AML, head and neck squamous cell carcinoma (HNSCC), oesophageal and gynaecological cancer (Butturini *et al.*, 1994; Rosenberg *et al.*, 2003); and mutations in *BRCA1* and *BRCA2*, which play critical roles in HRR, and are causally linked to breast and ovarian cancer and are also associated with prostate, pancreatic and other

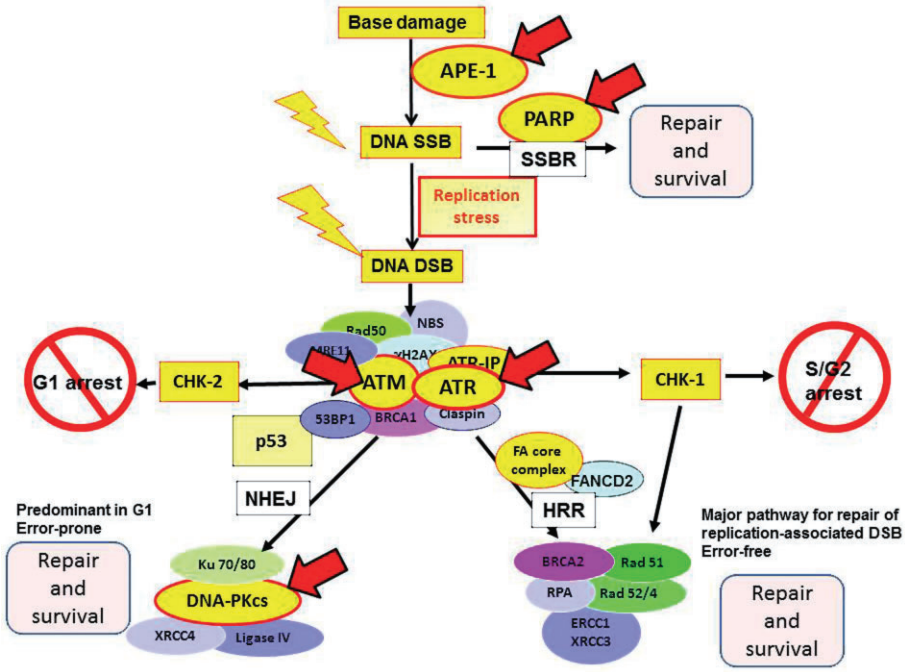

Figure 1

The DNA damage response (DDR). Simplified view of the cellular response to DNA damage. Base damage and DNA single-strand breaks (SSBs) are repaired by BER, in which APE-1 and PARP are key components. Unrepaired SSBs cause replication stress and DNA double-strand breaks (DSBs) in S-phase. DSB may also be induced directly. ATM and ATR signal the damage to cell cycle checkpoints and repair is either by non-homologous end-joining (NHEJ) or homologous recombination (HRR). DDR targets described in this review are indicated by red arrows.

gastrointestinal (GI) and gynaecological cancers, melanoma and hematopoietic cancers (Berman *et al.*, 1996; Brose *et al.*, 2002).

Loss of one element of the DDR may be compensated for by the activity of a complementary element of the DDR, which may be up-regulated as a result. Because the cancer cell is dependent on the compensatory DDR element, this has been termed non-oncogene addiction. This represents a vulnerability of the tumour cell that may be exploited by inhibitors of the DDR element to which it is addicted. This is the practical application of the principle of 'synthetic lethality', a term used to describe two events (mutations/inactivation/inhibition) that individually do not compromise survival, but together are lethal. On the other hand, up-regulation of elements of the DDR can result in resistance to DNA damaging chemo- and radiotherapy, and inhibitors of the DDR can therefore abrogate this mechanism of resistance. Thus, the rationale for the development of inhibitors of the DDR is twofold: firstly, to exploit cancer-specific defects in the DDR by the principle of synthetic lethality and, secondly, to overcome therapeutic resistance to DNA damaging chemo- and radiotherapy (Figure 2).

Targeting single-strand damage repair

The most common type of damage is damage to the nucleobases, largely in the form of oxidative damage due to the abundance of reactive oxygen species (ROS) arising from normal metabolic activity, and also spontaneous deamination and aberrant methylation due to the over-enthusiastic activity of *S*-adenosyl methionine and methyl transferase enzymes. This class of lesion is reported to occur at a frequency of 10^4–10^5 cell^{-1}·day^{-1} (Lindahl, 1993).

Direct repair

The simplest type of repair is direct repair, which simply reverses the lesion. Methylguanine DNA methyltransferase (MGMT) reverses the methylation of the O^6 position of guanine. Guanine methylation occurs naturally due to erroneous methylation and therapeutically following treatment with DNA alkylating agents such as the DNA methylating agents; temozolomide (TMZ) and DTIC (dacarbazine) and the bifunctional nitrogen mustards and nitrosoureas. High MGMT expression is associated with resistance to 1,3-bis (2-chloroethyl)-1 nitrosourea (BCNU) and TMZ (Pegg, 1990) in tumours, and pre-clinical studies demonstrated a strong correlation between MGMT activity and resistance to alkylating agents. MGMT is both transferase and acceptor; the reaction is stoichiometric and leads to a conformational change in the protein, targeting it for degradation (Ayi *et al.*, 1992). To regenerate MGMT activity, the protein must be re-synthesized. The higher levels in tumour tissue compared with normal tissue, coupled with the observation that MGMT$^{-/-}$ mice were viable and fertile and showed no increase in spontaneous tumourigenesis (although they are very sensitive to DNA methylating agents; Tsuzuki *et al.* 1996), suggested that artificial depletion of MGMT with pseudo-

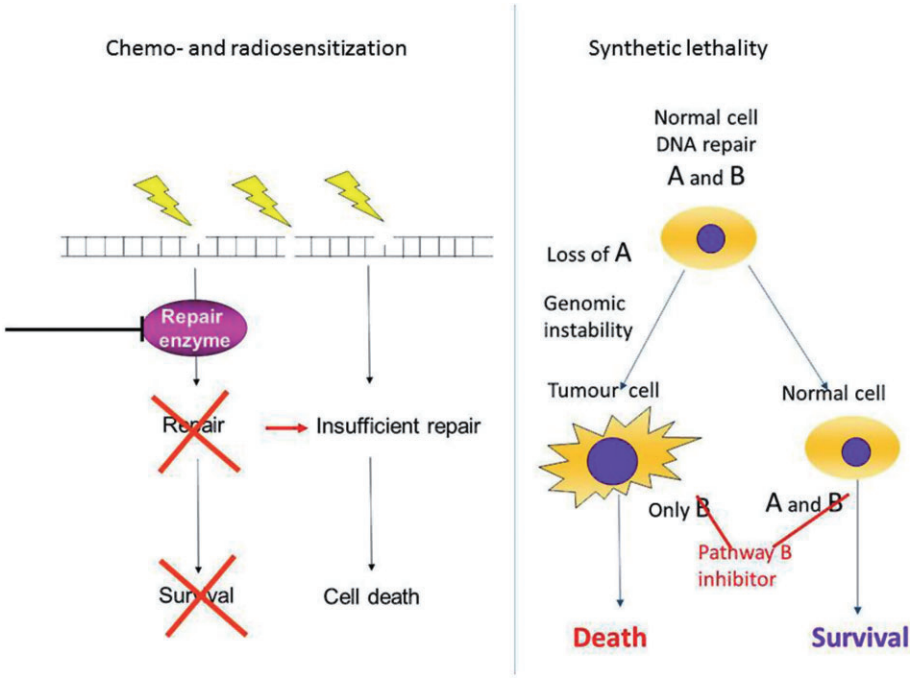

Figure 2

The rationale for the development of DNA damage response inhibitors. For chemo- and radiosensitization (left panel), the rationale is that by inhibiting repair, DNA damage that would otherwise be repairable and survivable persists, causing cell death. For synthetic lethality, the rationale is that there may be two repair components, A and B, that complement each other in a kind of functional redundancy. Loss of one pathway (A) creates the genomic instability that enables cancer to develop, but the cell is now completely reliant on the other component (B) such that inhibition of B causes the death of the tumour cell but not the normal cell, which still retains repair component A.

substrates might be a viable strategy to sensitize tumour cells, or at least create a 'level playing field'.

The preferred substrate of MGMT is O^6-methylguanine in double-stranded DNA, but other derivatives of guanine, alkylated at the O^6 position, also deplete MGMT (Figure 3). Free O^6-methylguanine was the first to be developed, but it was weak and poorly soluble. O^6-benzylguanine (BG), developed in the 1990s, was around 2000 times more potent than O^6-methylguanine, with an EC_{50} of 0.2 μM. Depletion of MGMT by pre-exposure to BG substantially increased CCNU cytotoxicity in human colon cells *in vitro* (Dolan *et al.*, 1990). Pre-clinical studies demonstrated that BG is metabolized to 8-oxo-0^6BG, which has similar potency and longer half-life (Dolan *et al.*, 1998). Furthermore, pharmacodynamic studies showed that BG depleted MGMT in tumour and normal tissues in mice-bearing human tumour xenografts and increased the anti-tumour activity of TMZ and BCNU (reviewed in Rabik *et al.*, 2006), although with a concomitant increase in bone marrow toxicity. Other MGMT pseudo-substrate inactivators have been developed with O^6-(4-bromothenyl)guanine (PaTrin-2, Lomeguatrib), a compound that is 10 times more potent than O^6-BG, showing sufficiently promising activity in pre-clinical studies to justify clinical evaluation (Middleton *et al.*, 2000; McElhinney *et al.*, 2003) (Figure 3).

The first clinical trial with BG was reported in 1998 (Friedman *et al.*, 1998). As a single agent, it was non-toxic at doses (120 $mg \cdot m^{-2}$) that depleted MGMT in tumour tissue, including gliomas, confirming its ability to cross the blood–brain barrier. Unfortunately, TMZ and BCNU-induced myelosuppression was also increased and substantial reductions of the primary cytotoxic dose were required (reviewed in Gerson, 2004). Several phase II trials using different schedules and routes of administration have been conducted, but generally they have shown only marginal clinical benefit, associated with toxicities, and the drug has not progressed to phase III trials. Lomeguatrib has also undergone clinical evaluation, and the first reported trial showed that it inhibited AGT in lymphocytes and tumour biopsies at a non-toxic dose of 10 $mg \cdot m^{-2}$. It only reduced the maximum tolerated dose (MTD) of temozolomide by 25% and there were some initial responses (Ranson *et al.*, 2006). However, subsequent phase II trials with 10 $mg \cdot m^{-2}$ lomeguatrib in combination with TMZ failed to show any substantial activity in melanoma or colon cancer patients, and this MGMT inactivator has also not progressed to advanced clinical trial (Khan *et al.*, 2008; Kefford *et al.*, 2009).

Base excision repair/single-strand break repair

The most common type of base damage (oxidation, deamination and methylation other than O^6-methylguanine) is repaired by the BER, in which the damaged base is removed and the abasic site is cleaved. The resultant single-strand break is then repaired by the rest of the SSBR pathway, which also deals with nicks in the deoxyribose backbone induced by ROS and the trapping of topoisomerase I-DNA complexes. Thus, the SSBR pathway acts downstream of a SSB; however, it is formed. Strictly speaking, BER refers to the excision of the

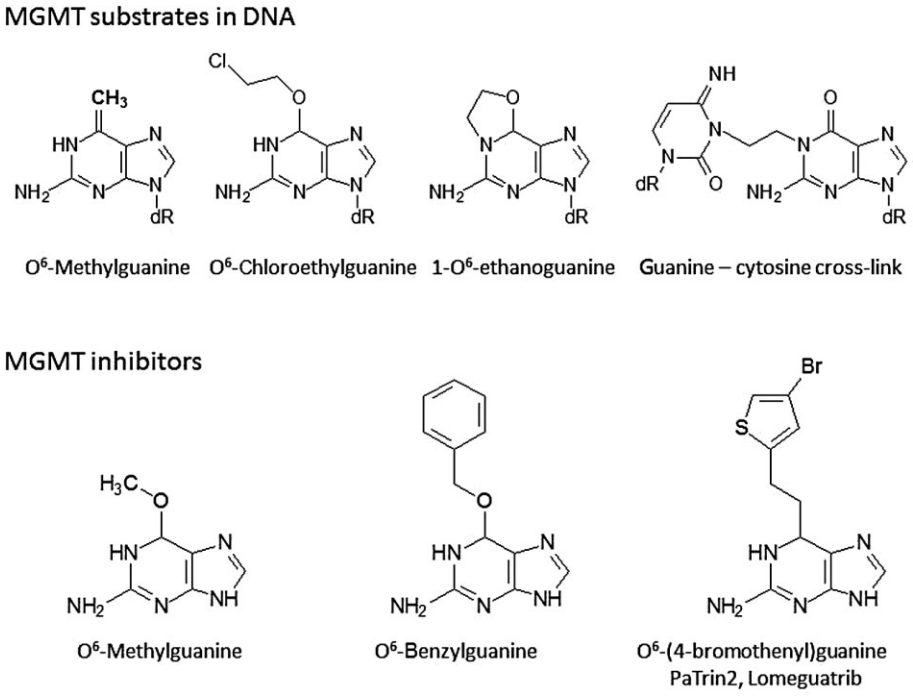

Figure 3

Methylguanine DNA methyltransferase (MGMT) substrates and inhibitors.

base and the SSBR pathway downstream of base excision, but not other forms of nick. However, in practice, the entire pathway is usually referred to as BER.

BER is subdivided into short- and long-patch repair corresponding to the removal and replacement of a single nucleotide or 1–13 nucleotides respectively. The pathway operates throughout the cell cycle; in the first step, the oxidized, deaminated and alkylated bases are removed by specific glycosylases that bind DNA and flip out the affected base from the minor groove. Endogenous and therapeutically induced alkylation depends on alkyladenine DNA glycosylase for this step (O'Connor and Laval, 1991). The resulting AP site is then hydrolysed by an AP endonuclease, with the major ones being AP endonuclease-1 (APE-1 aka Ref-1 or HAP-1) or AP lyase. The nick in the DNA is then repaired by short-patch BER or long-patch BER, depending on the nature of the 5' and 3' ends and, possibly, ATP availability. Short-patch BER is the predominant pathway. Polynucleotide kinase phosphatase (PNKP; a 3'DNA phosphatase and 5'DNA kinase), which may be necessary to modify the broken ends for replacement and/or rejoining, is most closely associated with BER. PCNA, 9-1-1 and Fen-1 are required for the processing of long patches. In short-patch repair, the single nucleotide is replaced by pol β and the gap is rejoined by ligase IIIα, and in long-patch repair, up to 13 nucleotides are replaced by pol δ/ε and rejoining is completed by ligase I (Cox *et al.*, 1996; Petermann *et al.*, 2003; Almeida and Sobol, 2007). PARP-1 (and/or PARP-2) and XRCC1 facilitate the repair by recruiting repair enzymes and providing the scaffold for short- and long-patch BER and the SSBR parallel pathway.

Therapeutically induced base damage following exposure to DNA methylating agents (methylation at N-7 position of guanine and N-3 position of adenine), IR (oxidative damage, principally 8-oxo-guanine) and fraudulent bases following anti-metabolite exposure as well as SSBs following IR-induced ROS generation and exposure to topoisomerase I poisons are all repaired by this pathway. Thus, the BER can contribute to the resistance to several anti-cancer agents, making it an attractive target for the development of inhibitors with the goal of achieving chemo- and radio-sensitization. Most studies have focused on inhibitors of APE-1 or PARP (Figure 4).

APE-1 inhibition. APE-1 is the major mammalian AP endonuclease and acts on abasic or 3'-blocking DNA lesions such as those generated by IR. Deletion of *APE-1* is embryonically lethal (Xanthoudakis *et al.*, 1996) and depletion of *APE-1* leads to the accumulation of AP sites and DNA breaks that inhibit proliferation and promotes cell death (Demple and Sung, 2005; Fishel *et al.*, 2007). High APE-1 is associated with drug and radiotherapy resistance; it is elevated in several tumour types and inactivation of APE-1, in the laboratory setting, confers sensitivity to IR and alkylating agents, making APE-1 inhibitors desirable for cancer therapy (Fishel and Kelley, 2007; Abbotts and Madhusudan, 2010). They potentiate DNA methylating agents such as TMZ and agents that lead to the misincorporation of fraudulent nucleotides. Such agents include the antifolate inhibitors of thymidylate synthase, for example, pemetrexed, that deplete the cells of thymidine nucleotides and lead to the misincorporation of uracil. There are two classes of inhibitor – methoxyamine (MX), which binds the AP site blocking the access of APE-1 to the site, and inhibitors of APE-1 endonuclease activity. Preclinically MX increased TMZ-induced DNA breaks and poten-

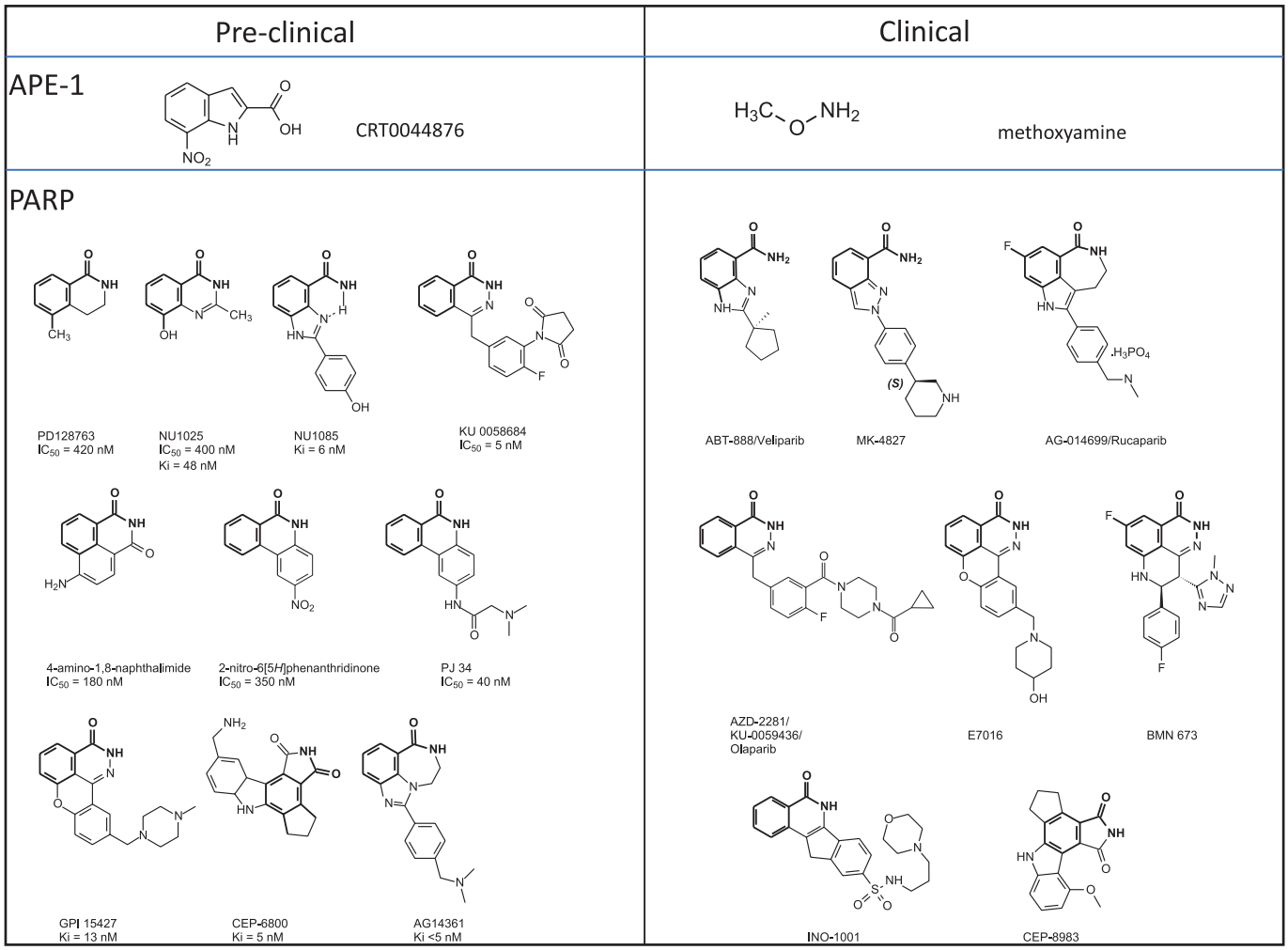

Figure 4

Base excision repair inhibitors. Pre-clinical inhibitors are shown on the left; those that have entered clinical evaluation are shown on the right. The two APR-1 inhibitors are shown above the line; the remaining inhibitors are PARPi (note the nicotinamide pharmacophore in these compounds).

tiated TMZ cytotoxicity (Taverna *et al.*, 2001). A study in ovarian cancer cells revealed that potentiation of TMZ was p53 independent, and MX also showed very impressive activity against human colon cancer xenografts (Liu *et al.*, 2002; Fishel *et al.*, 2007). MX also enhanced the radiosensitization by iododeoxyuridine (IUdR), which was thought to be due to increased incorporation or persistence of IUdR in the DNA and hence greater radiosensitization (Taverna *et al.*, 2003). Promising pre-clinical activity has been documented using MX and pemetrexed combinations, and the mechanism is related to uracil misincorporation and its excision by uracil glycosylase (Bulgar *et al.*, 2012). MX (TRC102) is now undergoing clinical evaluation in several trials in patients with advanced refractory cancers. A phase I trial in combination with pemetrexed has been reported with 14 out of 25 (including 4/5 NSCLC) patients showing a response at tolerable doses, and there is an ongoing study with TMZ (Weiss *et al.*, 2010).

Inhibitors of the endonuclease activity of APE-1 have also been identified, for example, lucanthone (a topo II inhibitor), which potentiates DNA methylating agents in breast cancer cells and showed radiosensitization activity in patients with brain metastases (Del Rowe *et al.*, 1999; Luo and Kelley, 2004). The development of inhibitors of APE-1 endonuclease activity has been facilitated by *in silico* modelling and high-throughput screening (HTS) using fluorescence quenching (Madhusudan *et al.*, 2005). The agents identified, including CRT0044876 (7-nitroindole-2-carboxylic acid), increased the persistence of AP sites generated and the cytotoxicity following alkylating agent treatment (Luo and Kelley, 2004; Bapat *et al.*, 2010; Wilson and Simeonov, 2010). These agents have not yet moved into advanced pre-clinical or clinical evaluation.

PARP inhibition. An alternative approach to targeting the BER pathway is to inhibit the activity of PARP-1 and PARP-2, which act downstream of the SSB to signal the break and recruit the repair enzymes. PARP-1 is the founding, most abundant and best characterized of a superfamily of PARP enzymes that have been identified by sequence homology

with the evolutionary highly conserved *PARP-1* catalytic domain, the 'PARP signature' (de Murcia and Ménissier de Murcia, 1994; Schreiber *et al.*, 2006). Its major role is thought to be in SSBR, but it is clear that it is also important in DNA DSB repair (Mitchell *et al.*, 2009). PARP-2 has somewhat overlapping functions with PARP-1. All bona fide PARPs catalyse the cleavage of NAD^+, releasing nicotinamide and catalysing the formation of homopolymers of ADP-ribose. PARP-1 has modest basal activity in the absence of DNA damage, but binding to DNA breaks via its zinc fingers activates PARP-1 to form long and branched ADP-ribose polymers attached largely to PARP-1 itself and histone H1. The high negative charge in the vicinity of the break is necessary for the recruitment of XRCC1 (El-Khamisy *et al.*, 2003), which, in turn, recruits PNPK and DNA polymerase, and the loosening of chromatin to facilitate repair.

Like APE-1, PARP activity is also significantly higher in tumour tissue compared with normal tissues, and PARP is a very attractive target to sensitize tumours to DNA damage that depends on SSBR for its repair. PARP inhibition by the prototype PARP inhibitor, 3-aminobenzamide (3AB), was first shown to retard the repair of strand breaks and reduce survival following exposure to DNA methylating agents in 1980 (Durkacz *et al.*, 1980).

The development of PARP inhibitors (PARPi) has been based on the observation that the by-product of NAD^+ cleavage by PARP, nicotinamide, is itself a weak PARP inhibitor, and most PARP inhibitors today have the nicotinamide pharmacophore incorporated into their structure. Although lacking sufficient potency and specificity for *in vivo* evaluation, 3AB provided 'proof of principle' data and remains a tool for PARP research. Research during the 1990s led to the development of more potent and specific second-generation inhibitors, including NU1025 and PD128763 (Suto *et al.*, 1991; Griffin *et al.*, 1995). During the same period, screening of commercially available compounds in an 'analogue by catalogue' approach, Banasik *et al.* (1992) identified several potent PARP inhibitors, including isoquinolinones and quinazolinones, which have been used as leads for further PARP inhibitor development. The most potent inhibitors had the carboxamide group constrained by incorporation into a ring structure or by intramolecular hydrogen bonding. Co-crystalization studies with the PARP inhibitors PD128763, 4-amino-naphthalimide and NU1025 in the NAD^+ binding site of the PARP-1 catalytic domain revealed important interactions of the carboxamide with critical amino acids, Ser904-OG and the Gly863-N, in the catalytic domain (Ruf *et al.*, 1996; 1998). Using the structural biology to direct chemical synthesis led to several highly potent inhibitors in which the carboxamide group was incorporated into a 7-membered ring (Canan Koch *et al.*, 2002; Calabrese *et al.*, 2003; 2004; Skalitzky *et al.*, 2003). These compounds included AG-014699 (rucaparib) (Thomas *et al.*, 2007) with a Ki of 1.4 nM that was the first PARP inhibitor to enter clinical trial for cancer patients (Plummer *et al.*, 2008). Several other PARP inhibitors have entered clinical investigation such as veliparib (ABT-888), which has also low nM Ki against both PARP-1 and PARP-2 (Penning *et al.*, 2009) and olaparib (AZD2281) with nM IC_{50} values against PARP-1 and PARP-2 (Menear *et al.*, 2008) (reviewed in Ferraris, 2010; Javle and Curtin, 2011).

Potentiation of DNA methylating agents. Pre-clinical studies with the more potent PARPi revealed that they potentiated the cytotoxicity and anti-tumour activity of DNA methylating agents, for example, TMZ, topoisomerase I poisons and ionizing radiation, with these observations being confirmed by genetic manipulation (reviewed in Jagtap and Szabo, 2005; Ferraris, 2010; Rouleau *et al.*, 2010; Javle and Curtin, 2011). Monofunctional alkylating agents are the most potent activators of PARP, and several studies have investigated TMZ chemosensitization by PARPi (reviewed in Tentori *et al.*, 2002; Curtin, 2005; Ferraris, 2010). Chemosensitization was not dependent on p53 status or tissue type (Delaney *et al.*, 2000). Interestingly, ABT-888 preferentially enhanced TMZ cytotoxicity during S-phase, indicating that replication-associated lesions are the most cytotoxic (Liu *et al.*, 2008). Loss of MMR is a major mechanism of cellular resistance to TMZ. Various PARP inhibitors (3AB, PD128763, NU1025, AG14361 INO-1001 and ABT-888) enhanced TMZ cytotoxicity preferentially in MMR-deficient cells and, in some cases, xenografts, completely overcoming MMR-mediated resistance (Wedge *et al.*, 1996; Tentori *et al.*, 1999; Curtin *et al.*, 2004; Cheng *et al.*, 2005; Horton *et al.*, 2009). Because only tumours lack MMR, PARP inhibition, in combination with TMZ, represents a potentially selective therapeutic approach.

The most extensive pre-clinical data are on ABT-888, which potentiated TMZ-induced tumour growth delay in a variety of subcutaneous, orthotopic and metastatic xenograft models of some of the most common and difficult to treat human cancers (Palma *et al.*, 2009). ABT-888 crosses the blood–brain barrier and significantly enhanced the anti-tumour activity of TMZ against intracranial human primary glioblastoma and in models of breast cancer brain metastases (Donawho *et al.*, 2007; Clarke *et al.*, 2009; Palma *et al.*, 2009). The enhancement of TMZ-induced tumour growth delay has also been investigated using other PARPi. In xenograft models of paediatric cancers, neuroblastoma and medulloblastoma, AG-014699 (rucaparib) increased TMZ activity (Daniel *et al.*, 2009; 2010). Complete tumour regressions have been observed in mice bearing U251MG (human glioblastoma) tumours treated with TMZ and CEP-6800 (Miknyoczki *et al.*, 2003) and in SW620 (human colon cancer) xenografts treated with TMZ in combination with AG14361 and AG14447 (Calabrese *et al.*, 2004; Thomas *et al.*, 2007). It was these latter studies that led to the first anti-cancer clinical trial of a PARP inhibitor (the phosphate salt of AG14447: AG014699/rucaparib) in 2003.

Potentiation of topoisomerase I poisons. Topoisomerase I is an essential enzyme that makes a single-stranded nick in the DNA, unwinds it and rejoins the ends to relieve the torsional stress generated during replication and translation. It can become trapped on DNA by clustered ROS damage or by one of the topoisomerase I poisons, for example, camptothecin, irinotecan or topotecan, part-way through the process generating a protein-associated single-strand break. Topoisomerase I is removed from the break by tyrosyl DNA phosphodiesterase (Tdp1), generating 3'phosphate and 5'hydroxyl ends that are dephosphorylated and phosphorylated, respectively, by PNPK. PARP and XRCC1 are required for this process (Plo *et al.*, 2003). Poly(ADP-ribosyl)ated PARP-1 and PARP-2, but

not the unmodified enzymes, may also accelerate the removal of camptothecin-stabilized topoisomerase I–DNA cleavable complexes (Malanga and Althaus, 2004).

Several studies have investigated the therapeutic potential of PARP inhibitors in combination with topoisomerase I poisons. Sensitization is generally modest (two- to threefold) compared to the DNA methylating agents (more than fivefold). Initial studies revealed that NU1025 markedly increased camptothecin-induced DNA breaks and that both NU1025 and the more potent PARPi, NU1085, potentiated topoisomerase 1 poison-induced cytotoxicity in a panel of human cancer cell lines (Delaney *et al.*, 2000; Bowman *et al.*, 2001). Subsequently, repair of camptothecin-induced DNA breaks was found to be slower in PARP-1 knockout cells and in human leukaemic cells treatment with AG14361 and AG14361 in increased topoisomerase I-induced cytotoxicity (Smith *et al.*, 2005). Similarly, ABT-888 increased the persistence of topoisomerase I poison-induced DNA breaks and enhanced cytotoxicity (Patel *et al.*, 2012). AG14361 potentiated topotecan-induced growth inhibition in human colon and lung cancer cells (Calabrese *et al.*, 2004). AG14447 (the parent compound of AG-014699, rucaparib), increased topotecan cytotoxicity and GPI 15427 increased SN-38 (the active metabolite of irinotecan) cytotoxicity in human colon cancer cell lines (Tentori *et al.*, 2006; Thomas *et al.*, 2007). Sensitization of xenografts to topoisomerase I poisons has also been demonstrated with CEP-6800 (Miknyoczki *et al.*, 2003), AG14361 and GPI15427 (Calabrese *et al.*, 2004; Tentori *et al.*, 2006), and AG-014699 (rucaparib) (Daniel *et al.*, 2009).

Potentiation of ionizing radiation. The first evidence that PARPi are radiosensitizers came from a study with 3AB, which increased IR-induced cytotoxicity in mammalian cells (Ben-Hur *et al.*, 1985). These initial studies have been confirmed by radiosensitization studies using a variety of PARPi (ANI, NU1025, AZD2281, E7016) in multiple cell line models with dose-enhancement ratios of 1.3–1.7 (Bowman *et al.*, 1998; Schlicker *et al.*, 1999; Brock *et al.*, 2004; Dungey *et al.*, 2008; Russo *et al.*, 2009). PARPi have been shown to radiosensitize replicating cells (Banasik *et al.*, 1992), possibly by increasing the persistence of SSBs that convert to DSBs upon collision with replication forks (Saleh-Gohari *et al.*, 2005). This would be consistent with the observed persistence of IR-induced DSB and stimulation of homologous recombination following PARPi treatment (AZD2281/olaparib and E7016) (Dungey *et al.*, 2008; Russo *et al.*, 2009; Harper *et al.*, 2010). Alternative studies in growth arrested cells indicate that PARP inhibition inhibits the recovery from potentially lethal IR (Calabrese *et al.*, 2004; Thomas *et al.*, 2007).

In vivo studies revealed that PD128763 increased the therapeutic effect of X-rays up to threefold against sarcoma xenografts (Leopold and Sebolt-Leopold, 1992); AG14361 increased the efficacy of fractionated X-rays against human colon cancer xenografts (Calabrese *et al.*, 2004); ABT-888 (veliparib) increased the anti-tumour activity of IR in xenograft models of human colon, lung and prostate cancer (Albert *et al.*, 2007; Donawho *et al.*, 2007; Barreto-Andrade *et al.*, 2011); GPI15427 increased the radiosensitivity of HNSCC xenografts (Khan *et al.*, 2010); MK-4827 radiosensitized human lung and breast carcinoma xenografts (Wang *et al.*, 2012); and AZD-2281 (olaparib) radiosensitized small-cell lung cancer xenografts (Senra *et al.*, 2011). ABT-888 also significantly potentiated the combination of TMZ and IR in mice bearing intracranial gliomas (Clarke *et al.*, 2009). Some of the radiosensitization may be due to the vasoactive effect of the PARP inhibitors, which has been demonstrated for AG14361, AG-014699 (rucaparib) and AZD-2281 (olaparib) (Ali *et al.*, 2009; 2011; Senra *et al.*, 2011).

Clinical studies with PARPi. The highly encouraging activity seen in combination with TMZ in xenograft studies (Calabrese *et al.*, 2004; Thomas *et al.*, 2007) led to the first clinical trial of a PARP inhibitor (AG-014699, CO-338, rucaparib) in combination with TMZ in 2003 (Plummer *et al.*, 2008). In this study, a dose-dependent increase in PARP inhibition in surrogate normal tissues (peripheral blood mononuclear cells, PBMCs) and tumour biopsies was observed, and a PARP-inhibitory dose of 12 $mg{\cdot}m^{-2}$ was not toxic with full-dose TMZ. Myelosuppression was observed in subsequent phase II studies of the combination in melanoma patients, requiring dose reductions (Plummer *et al.*, 2006). Nevertheless, despite the reduced dose of TMZ, the study reported an increase in the response rate and median time to progression compared to historical reports of TMZ alone. In contrast to these data, olaparib did not improve the response to dacarbazine (DTIC, a closely related drug to TMZ) (Khan *et al.*, 2011), and dose-limiting myelosuppression was observed with the combination of INO-101 with TMZ (Bedikian *et al.*, 2009). Studies in combination with topoisomerase I poisons have also identified serious toxicities. Profound myelosuppression was seen in a phase I study of ABT-888 (veliparib) with topotecan, which was ameliorated by revising the doses and schedules and the MTD was established as topotecan 0.6 $mg{\cdot}m^{-2}{\cdot}day^{-1}$ with ABT-888 10 mg. In this study, PARP activity was reduced in both tumour and PBMCs and, importantly, increased DNA breaks were detected in circulating tumour cells and PBMCs, and some disease stabilization was observed (Kummar *et al.*, 2011). Dose-limiting diarrhoea and neutropenia was observed in a study of veliparib (ABT-888) in combination with irinotecan (LoRusso *et al.*, 2011), and dose-limiting neutropenia and thrombocytopenia was seen in a phase I study of olaparib and topotecan at low doses (Samol *et al.*, 2011). Using a targeted therapy, such as radiotherapy, may result in fewer side effects. Radiotherapy trials with PARPi have been initiated, but to date, there are no final reports. An interim report, published in abstract form, showed that up to 200 mg ABT-888 (veliparib) twice daily was well tolerated in combination with whole brain radiotherapy in patients with brain metastases from advanced solid tumours (Mehta *et al.*, 2012). A summary of the clinical trials with PARP inhibitors is given in Table 1. In addition, there are several studies of PARPi combinations that have not been substantiated by peer-reviewed pre-clinical data, including combinations with doxorubicin (pegylated or otherwise) pemetrexed, gemcitabine, epirubicin, cyclophosphamide, vinorelbine, neratinib, filigastrim, pegrastim, 5FU or capcitabine and, most irrational of all, paclitaxel (see http://www.clinicaltrials.gov); it will be interesting to see if there are any responses to these combinations not predicted by the pre-clinical data.

Table 1

PARP inhibitors in clinical trial

Date started	Name Company	Single agent/combinations	Tumour type	Route Current stage
2003	AG-014699 CO-338 Rucaparib Pfizer Now Clovis Oncology	TMZ combination Various combinations Single agent	Solid tumours Melanoma BRCA mutant breast ovarian	i.v. p.o. Phase II
2003/6	INO-1001 Inotek/Genentek	TMZ combinations	Melanoma	i.v. Phase I (terminated)
2005	KU59436/AZD2281 Olaparib AstraZeneca	Single agent Various combinations	Solid tumours BRCA carriers TNBC/HGSOC Solid tumours	p.o. Phase II
2006	ABT-888 Veliparib Abbott	Single agent Various combinations	Various solid + Lymphoblastoid	p.o. Phase II
2008	MK4827 Merck	Single agent Combinations with TMZ or doxorubicin	Solid and haematological tumours GBM, ovarian	p.o. Phase II Phase I
2009	CEP-9722 Cephalon	Single agent Combination with TMZ Gem/cis	Solid tumours Lymphoma	p.o. Phase I
2010	GPI21016/E7016 MGIPharma/Eisai	Combination with TMZ	Melanoma	p.o. Phase II
2011	BMN-673 BioMarin	Single agent	Various solid and haematological tumours	p.o. Phase I

Synthetic lethality with PARPi. The most exciting development in the field of DNA repair inhibition for anti-cancer therapy is the application of synthetic lethality by targeting the remaining complementary pathway on which DNA repair-defective cancer cells are dependent. This has been elegantly demonstrated by using PARPi to treat HRR-defective cancers (Bryant *et al.*, 2005; Farmer *et al.*, 2005). The hypothesis that PARP inhibition might be synthetically lethal in HRR-defective cells originally came from the observation that loss or inhibition of PARP resulted in a hyper-recombinogenic phenotype, characterized by increased RAD51 focus formation and chromosomal rearrangements. This suggested that failure to repair the high level of DNA SSbreaks by PARP-dependent mechanisms resulted in an accumulation of stalled replication forks requiring HRR for their resolution (Schultz *et al.*, 2003) (Figure 5). These observations were important because defects in aspects of HRR are common in cancer, with the classic example of *BRCA1* and *2* defects and their association with breast and ovarian cancer syndrome (Venkitaraman, 2002). Further studies reveal that knockdown of various other genes involved in HRR also confer sensitivity to PARP inhibitors (McCabe *et al.*, 2006) and opened up the possibility in targeting the tumour cells without toxicity to the normal tissues. Surprisingly, in an siRNA screen, *PTEN* and *CDK5* knockdown were also identified as being synthetically lethal with PARP inhibition; CDK5 is involved in checkpoint signalling and PTEN may regulate RAD51 function (Shen *et al.*, 2007; Lord *et al.*, 2008; Turner *et al.*, 2008). *PTEN* is a tumour suppressor gene that is commonly mutated in cancer and *PTEN* mutant cells were killed by single agent olaparib (Mendes-Pereira *et al.*, 2009). However, further studies suggest that this may not be universally applicable as *PTEN* deletion was not associated with defective RAD51 expression or marked hypersensitivity to PARPi in prostate cancer cells (Fraser *et al.*, 2012).

The PARPi AG-014699 (rucaparib) showed good single-agent activity in a panel of human cancer cells and xenografts with defects in *BRCA1* or *BRCA2* that included human breast cancer cell line with epigenetic silencing of *BRCA1*, rather than a mutation, demonstrating the potential in sporadic cancer (Drew *et al.*, 2011a). One critical observation from the pre-clinical studies is that much higher doses and prolonged exposures are required for single-agent PARPi activity against HRR-defective cells and xenografts than for chemo- and radiosensitization, and that PARPi doses and schedules that are non-toxic as single agents are lethal in combination with a cytotoxic. To take AG-014699 (rucaparib) as an example, profound chemo- and radiosensitization was achieved *in vitro* with sub-micromolar concentrations and *in vivo* chemosensitization, resulting in complete tumour regression, was observed with 1 $mg{\cdot}kg^{-1}$ daily ×5, which was the MTD in combination with TMZ (Thomas *et al.*, 2007). In contrast, concentrations of rucaparib as a single agent up to 7 μM were needed to kill 50% of BRCA defective human cancer *cells in vitro* and to achieve inhibition of tumour growth *in vivo* doses ≥10 $mg{\cdot}kg^{-1}$ for ≥10 days were required and doses as high as 50 $mg{\cdot}kg^{-1}$ daily were completely non-toxic (Drew *et al.*, 2011a).

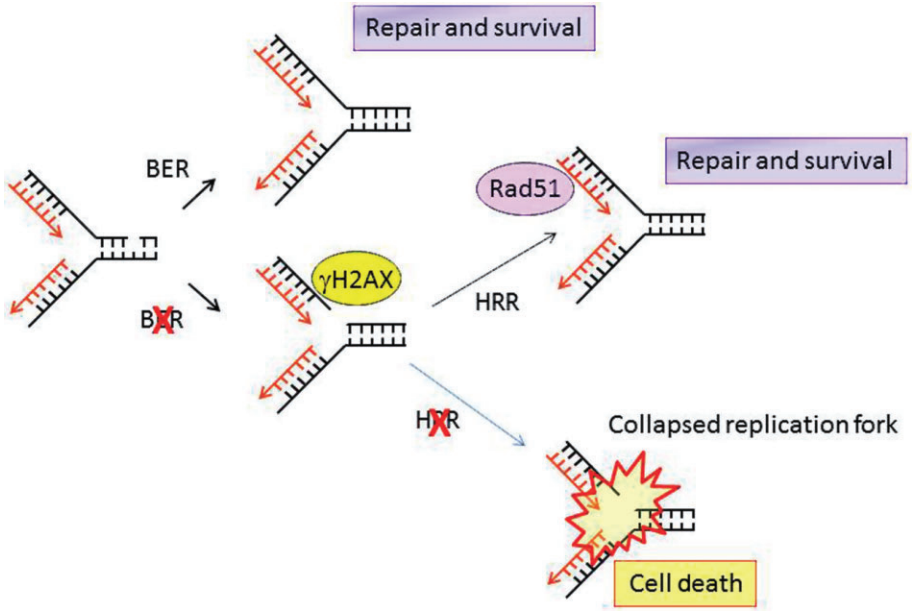

Figure 5

Synthetic lethality of PARP (base excision repair, BER) inhibitors in cells lacking homologous recombination repair (HRR). DNA ingle-strand breaks (SSBs) are repaired by BER, if unrepaired then on collision with the replication fork they form a collapsed replication fork and single-ended double-strand breaks (DSBs) (which may be identified by the accumulation of γH2AX). This lesion requires HRR to repair and re-start the fork (identified by RAD51 foci), but in a cell lacking HRR, the collapsed replication fork persists and the cell dies.

Interestingly, many HRR genes appear to be suppressed in hypoxic conditions, potentially making hypoxic cells more sensitive to PARP inhibition (Bindra *et al.*, 2005; Chan *et al.*, 2008). This 'contextual synthetic lethality' has been demonstrated *in vivo* with increased γH2AX and apoptosis and reduced RAD51 foci seen in hypoxic regions of xenografts in mice treated with a PARPi (Chan *et al.*, 2010). Further promising developments suggest that an HRR defect can be constructed using another targeted agent. The cyclin-dependent kinase, CDK1, phosphorylates BRCA1 enabling it to form repair foci and CDK1 inhibition leads to inactivation of BRCA1. CDK1 inhibition and PARP inhibition alone are non-toxic, but the combination of a CDK1 inhibitor and a PARPi was cytotoxic in lung cancer cells, xenografts and spontaneous lung tumours in genetically engineered mice, but spared normal tissues (Johnson *et al.*, 2011). However, resistance to PARPis can develop due to secondary mutations in *BRCA1* or *2* that restore their function (Edwards *et al.*, 2008; Sakai *et al.*, 2008; Swisher *et al.*, 2008). In addition, even in *BRCA1* mutant cells, HRR function and PARPi resistance can be restored if 53BP1 or DNA-PKcs (components of NHEJ) are also inactivated (Bouwman *et al.*, 2010; Bunting *et al.*, 2010; Patel *et al.*, 2011). This phenomenon has been called 'synthetic viability'. Loss of 53BP1 appears to be a relatively common event in *BRCA1* mutant and triple negative breast cancer, which could compromise the activity of PARP inhibitors in clinical trials against breast cancer (Bartkova *et al.*, 2007).

Nevertheless, the prospect of non-toxic therapy has real clinical potential and these experimental observations have provided a boost to PARP research with nine PARPis now undergoing clinical evaluation as single agent as well as in combination with conventional cytotoxic therapy (Table 1).

The first report of a clinical trial exploiting the principle of synthetic lethality was the pivotal phase I study using olaparib (Fong *et al.*, 2009a,b) enriched with patients carrying *BRCA1/2* mutations. Olaparib showed good oral bioavailability, was well tolerated with an MTD of 400 mg twice daily, and responses were reported in 12 of the 19 *BRCA1* and *2* mutation carriers, including patients with breast, ovarian and prostate cancer, but there were no responses in non-*BRCA* mutation carriers. Two parallel phase II studies were then undertaken with olaparib; one in patients with breast cancer and the other in patients with ovarian cancer with *BRCA 1* or *2* mutations. Patients received either 100 or 400 mg olaparib. The common adverse effects were mild, including fatigue, nausea and vomiting. In the patients on the 400 mg dose, the overall response rate in the breast cancer group was 41 and 33% in the ovarian group, but the response rate was lower at 100 mg in the breast group (22%) and ovarian group (12.5%), indicating a dose-dependency of the response (Audeh *et al.*, 2010; Tutt *et al.*, 2010). Other single-agent PARPi studies are underway and interim results of the phase II trial of single-agent rucaparib (AG-014699) in patients with *BRCA*-mutated breast and/or ovarian cancer reported a clinical benefit rate of 34% (Drew *et al.*, 2011b).

PARPi are also being evaluated in patients with non-germline *BRCA*-mutated cancers, particularly high-grade serous ovarian cancers (HGSOC) and triple negative breast cancer (TNBC). In a phase II study, the efficacy of continuous olaparib (400 mg twice daily) in HGSOC patients with or without known *BRCA* mutations and of *BRCA*-mutated breast cancer or TNBC patients with unknown *BRCA* status was compared (Gelmon *et al.*, 2011). Encouragingly, in the patients with non-germline BRCA mutated HGSOC, there

was a response rate of 24%, compared with 41% in the confirmed BRCA mutation ovarian cancer patientsm but, disappointingly, no responses were observed in the two breast cancer arms. This is the *first* study to show single-agent PARP inhibitor activity in non-germline BRCA mutated cancers, indicating that sporadic HGSOC could be targeted with PARP inhibitors.

Targeting double-strand break repair

A DNA DSB is much more cytotoxic and difficult to repair than SSBs. Cells therefore have, of necessity, developed complex mechanisms to repair DNA DSBs. There are two major DSB repair pathways: NHEJ and HRR. HRR is a high-fidelity repair pathway using the sister chromatid as a template and can therefore only function during S and G2 phases of the cell cycle, while NHEJ involves the simpler religation of the broken ends with minimal processing and is therefore more error-prone but active in all phases of cell cycle, predominating in G0/G1 (Shrivastav *et al.*, 2008).

Therapeutically induced DSBs result directly from exposure to IR and topoisomerase II poisons, and indirectly from the collision of the replication fork with the single-stranded lesion. Topoisomerase II poisons, which are used to treat nearly half of all cancers, lock topoisomerase II-DNA complex in the open gate conformation, creating a persistent protein-associated DSB (McClendon and Osheroff, 2007). IR induces approximately 1 DSB for every 25 SSB, but the radiomimetics bleomycin and neocarzinostatin produce a higher frequency of DSB (10 and 30% of the total breaks, respectively) (Dedon *et al.*, 1992; Povirk, 1996; Nikjoo *et al.*, 2001).

Targeting non-homologous end-joining

NHEJ is estimated to repair up to 85% of IR-induced DSBs (Rothkamm *et al.*, 2003; Mahaney *et al.*, 2009; Shibata *et al.*, 2011). The core NHEJ proteins are Ku 70/80, DNA-PKcs, Artemis XRCC4 ligase IV and XLF (XRCC4-like factor) (Figure 6). The Ku heterodimer binds DS DNA ends with no sequence requirements. This promotes the recruitment and activation of the DNA-PK catalytic subunit (DNA-PKcs) to form the DNA-PK trimeric holoenzyme necessary to bring about synapsis of the DNA ends. The formation of the holoenzyme on DNA stimulates the catalytic activity of DNA-PKcs. DNA-PKcs is a kinase in the PI3K family of kinases and its kinase activity is essential for NHEJ (Kurimasa *et al.*, 1999). DNA-PKcs phosphorylates H2AX and also itself, allowing dissociation (Merkle *et al.*, 2002; Mahaney *et al.*, 2009). Artemis processes the DNA ends and the final ligation of the juxtaposed ends is accomplished by ligase IV stabilized by the XRCC4/XLF complex (Lees-Miller and Meek, 2003; Ahnesorg *et al.*, 2006; Burma *et al.*, 2006). The critical role of NHEJ in

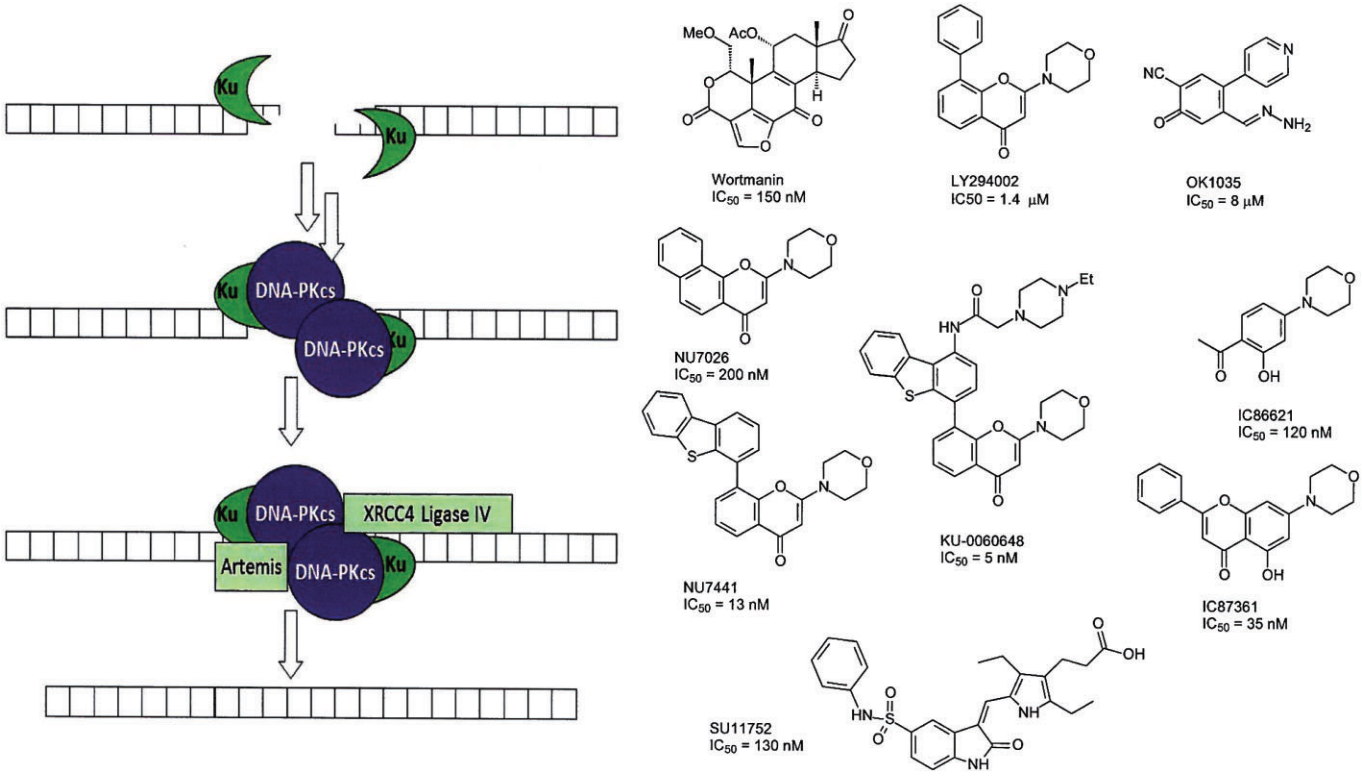

Figure 6

Non-homologous end-joining and DNA-PK inhibitors. The Ku heterodimer is recruited to the DSB and, in turn, recruit DNA-PKcs, the holoenzyme thus formed recruits Artemis and XRCC2 and ligase IV to further stabilize, process the ends and relegate them to restore DNA continuity. Various inhibitors described in the text are shown on the right hand side (note the morpholino group in many of the compounds).

DNA DSB repair was underscored by the demonstration that cells defective in NHEJ are sensitive to IR and topoisomerase II poisons (Jeggo *et al.*, 1989; Tanaka *et al.*, 1993). Thus, inhibition of DNA-PK is an attractive approach to modulating therapy resistance.

DNA-PK is a member of the PI3K-related protein kinase (PIKK) family of enzymes. Inhibitors of PI3K, such as wortmaninn and LY294002, are also non-competitive and competitive inhibitors of DNA-PK respectively (Izzard *et al.*, 1999). Both compounds retard DNA DSB rejoining and enhance the cytotoxicity of IR and the topoisomerase II poison, etoposide, most probably by inhibiting DNA-PKcs rather than the other PIKKs (Price and Youmell, 1996; Rosenzweig *et al.*, 1997; Boulton *et al.*, 2000). Using LY294002 as a lead, more potent and specific DNA-PK inhibitors have been developed, for example, NU7026, NU7441, IC86621 and IC87361 (Allen *et al.*, 2003; Leahy *et al.*, 2004; Hardcastle *et al.*, 2005; Shinohara *et al.*, 2005) (Figure 6). NU7441 was highly potent and specific, with an IC_{50} of only 14 nM and at least 100-fold selectivity for this enzyme compared with other PI3K family kinases. All of the inhibitors substantially slow down DSB repair and increase the cytotoxicity and anti-tumour activity of ionizing radiation, radiomimetics and topoisomerase II poisons in cells and xenografts in a variety of models and have been shown to be DNA-PK-specific by comparing their sensitization effects in cells with and without DNA-PKcs (Kashishian *et al.*, 2003; Veuger *et al.*, 2003; Willmore *et al.*, 2004; Zhao *et al.*, 2006). Another structurally different, but less potent, DNA-PK inhibitor, OK-1035, inhibited DNA repair in radioresistant L5178Y cells (Kruszewski *et al.*, 1998). SU11752 was identified by library screening as an ATP-competitive DNA-PK inhibitor with comparable potency to wortmaninn, but with selectivity for DNA-PK over PI3K and ATM. SU11752 profoundly inhibited DNA DSB repair and sensitized DNA-PK competent MO59J but not the defective MO59K cells to ionizing radiation but lacked sufficient potency for *in vivo* studies (Ismail *et al.*, 2004). However, some of the DNA-PK inhibitors have been investigated in tumour-bearing mice. IC86621 increased the IR-induced tumour growth delay and improved survival by fourfold in mice bearing human colon cancer xenografts (Kashishian *et al.*, 2003). Similarly, in mice-bearing human colon cancer, xenografts NU7441 increased etoposide-induced tumour growth delay by twofold (Zhao *et al.*, 2006). Recently, KU-0060648, a dual inhibitor of DNA-PK and PI3K, increased etoposide-induced tumour growth delay in mice bearing SW620 and MCF-7 xenografts by up to 4.5-fold, thus warranting further evaluation of joint DNA-PK and PI3K inhibitors (Munck *et al.*, 2012).

DNA-PKcs levels and activity were higher in poor prognosis patient-derived B-CLL samples and the DNA-PK inhibitors NU7026 and NU7441 enhanced their sensitivity to chlorambucil, fludarabine and various topoisomerase II poisons, including doxorubicin, etoposide and mitoxatrone (Willmore *et al.*, 2004; 2008; Elliott *et al.*, 2011). To date, the only DNA-PK inhibitor to have progressed to clinical trial is CC-115, a dual mTOR and DNA-PK inhibitor, which is undergoing phase I evaluation in multiple myeloma, non-Hodgkin's lymphoma and various solid tumour types, including Ewing's sarcomas. However, this study has not been reported yet.

Targeting HRR

The HRR pathway of DSB repair is highly complex and high fidelity as it uses the complementary DS DNA on the sister chromatid to act as a template for accurate re-synthesis of the damaged DNA. It can only operate during S and G2 and it is the predominant pathway in S/G2 (Shrivastav *et al.*, 2008). This is also the pathway that deals with stalled/collapsed replication forks and single-ended DSBs that result from the collision of the replication fork with a SSB or other lesion. It is inextricably linked to S and G2 checkpoints through the activity of ATM and ATR (Figure 7). HRR is a multi-step pathway in which the MRN complex (MRE11-Rad50-NBS1), facilitated by BRCA1, recognizes and cooperates with CtIP and EXO for end resection (Zhong *et al.*, 1999). MRN recruits and activates ATM (Paull and Lee, 2005). ATM, as well as DNA-PK, phosphorylates histone H2AX, and the accumulation of γH2AX at the site of the break aids the recruitment of 53BP1, RNF168 and BRCA1 (Stiff *et al.*, 2004; Derheimer and Kastan,

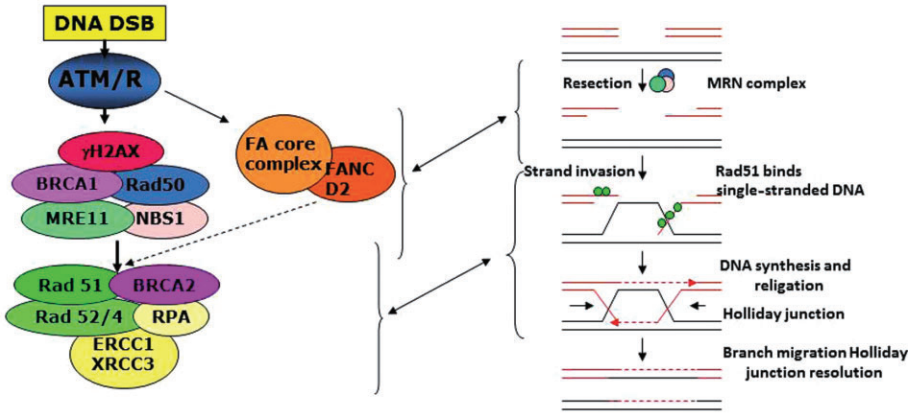

Figure 7

Homologous recombination repair (HRR). Recognition signalling and repair components are shown on the left, the mechanics of end resection, strand invasion, re-synthesis across the break, relegation and Holliday junction resolution are shown on the right.

2010). ATM phosphorylates and activates MRE11, NBS1, CtIP and EXO (Di Virgilio *et al.*, 2009; Bolderson *et al.*, 2010). The resulting long SS DNA overhang is rapidly coated with RPA, preventing its degradation. This recruits the ATRIP-ATR complex, which signals to Chk1 for S and G2 arrest. Stalled replication forks primarily activate ATR rather than ATM (Flynn and Zou, 2011). ATM and ATR both phosphorylate BRCA1 at multiple sites, stimulating its E3 ubiquitin ligase activity. BRCA1 ubiquitinates CtIP to activate the G2 checkpoint (Cortez *et al.*, 1999). ATR phosphorylates a number of targets to promote HRR, including RPA2 and Chk1, which, in turn, phosphorylates RAD51, both of which are needed for the formation of RAD51 foci (Sorensen *et al.*, 2005; Shi *et al.*, 2010). BRCA2, which also interacts with PALB2 and BRCA1 (Zhang *et al.*, 2009), delivers RAD51, which displaces the RPA to form the nucleoprotein filament that can invade the complementary duplex DNA (Jensen *et al.*, 2010; Liu *et al.*, 2010; Thorslund *et al.*, 2010). This displaces the other strand to form a 'D-loop', various RAD51 homologues (RAD51B, C, D and XRCC2 and 3) interact with the filament and RAD54 removes the RAD51 at later stages of HRR (Heyer *et al.*, 2006; Liu *et al.*, 2007). Once the invading strand has been annealed, it is extended by DNA polymerase to rejoin the SS DNA on the opposite end of the DSB, or the D loop can migrate further and anneal with the other end of the broken DNA (Sung and Klein, 2006). The Holliday junctions (where the DNA is crossed over) are resolved by HJ resolvases (MUS81-EME1, GEN1 and SLX1-SLX4) or BLM – TopoIII-RM11 (Bugreev *et al.*, 2008; Gari *et al.*, 2008; Ip *et al.*, 2008) (Figure 7).

The function of the entire repair pathway can be compromised if one or more genes involved in the pathway are mutated. As HR is the principal repair pathway during the S-phase of the cell cycle and is essential for error-free DNA repair, it is critical for the maintenance of genomic stability. Mutations in HRR genes are associated with cancer, classically, germline heterozygous mutations in BRCA1 and BRCA2 are causally linked to breast and ovarian cancer and are also associated with prostate, pancreatic and other GI and gynaecological cancers, melanoma and hematopoietic cancers (Berman *et al.*, 1996; Brose *et al.*, 2002), and methylation silencing of BRCA1 is associated with breast, ovarian and NSCLC (Dobrovic and Simpfendorfer, 1997; Esteller *et al.*, 2000; Lee *et al.*, 2007; Lahtz and Pfeifer, 2011). Homozygous mutation in *ATM* confers an approximately 100 times increased risk of cancer (Taylor *et al.*, 1975), with heterozygous *ATM* mutations also linked to an increased risk of cancer (Thompson *et al.*, 2005), and epigenetic silencing of *ATM* has been reported in breast and HNSCC (Ai *et al.*, 2004; Flanagan *et al.*, 2009). Point mutation in *MRE11* have been found in ovarian cancers, and shortening of the T(11) repeat microsatellite was detected in 93% of primary colorectal cancer (Giannini *et al.*, 2002; Heikkinen *et al.*, 2003). Similarly, frameshift mutations in the microsatellite in *RAD50*, resulting in a truncated protein, have been reported in 31% GI cancers (Kim *et al.*, 2001) and mutations in *NBS1* cause cancer predisposition (Digweed and Sperling, 2004). It would therefore seem paradoxical to develop inhibitors of HRR. However, HRR defects are associated with profound sensitivity to a variety of DNA damaging agents, for example, *ATM* and *NBS1* mutations confer hypersensitivity to ionizing radiation (Digweed and Sperling, 2004; Thompson *et al.*, 2005).

With the exception of mirin, which inhibits MRE11 (Dupré *et al.*, 2008) and some inhibitors of RAD51 recently identified by HTS (Huang *et al.*, 2011), there are no bona fide inhibitors of HRR, although there is some suggestion that imatinib can inhibit RAD51 focus formation (Choudhury *et al.*, 2009), PI3K inhibitors are reported to down-regulate BRCA1 and impair RAD51 focus formation (Ibrahim *et al.*, 2012; Juvekar *et al.*, 2012), and CDK1 inhibition can indirectly inhibit RAD51 focus formation by blocking CDK1-mediated BRCA1 phosphorylation (Johnson *et al.*, 2011). Most research has focused on signalling of DNA damage to cell cycle checkpoints via ATM and, more recently, ATR.

ATM inhibitors. ATM has been investigated as a target for cancer therapy, but there are limited studies with inhibitors due to the lack of availability of potent small-molecule inhibitors. Based on the structural similarity of ATM to PI3K, the first selective ATM inhibitor, KU55933, was developed from the PI3K inhibitor, LY294002 (Hollick *et al.*, 2007) (Figure 8). KU55933 inhibited the IR-induced ATM-dependent events, for example, p53 phosphorylation at serine 15 and sensitized cancer cells to IR and topoisomerase I and II inhibitors (Hickson *et al.*, 2004). Radiosensitization by KU55933 has been demonstrated in a variety of human cancer models, and its synergistic radio- and chemosensitization in combination with DNA-PK inhibition in prostate cancer cells (Shaheen *et al.*, 2011). Further elaboration of the core structure led to the development of KU-60019, with increased activity and being a more potent radiosensitizer (Golding *et al.*, 2009). CP466722 was identified as an ATM inhibitor by library screening. Studies with KU55933 and CP466722 demonstrated that inhibition of ATM activity for 4 h was sufficient for significant radiosensitization (Rainey *et al.*, 2008). ATM inhibition appears to be different from lack of ATM; repair of damaged DNA replication forks is normal in AT cells after IR but is inhibited by KU55933 in wild-type cells but not AT cells. This suggests that inhibited ATM physically impedes recombination at damaged replication forks via blocking CtIP-MRN end resection (Choi *et al.*, 2010).

ATR inhibitors. Loss of G1 checkpoint control is a common feature of cancer cells, for example, due to defects in the p53 and pRb tumour suppressor genes or an imbalance in cyclins, cyclin-dependent kinases and their inhibitors (reviewed in Sherr, 1996; Massague, 2004). This makes cancer cells more reliant on their S/G2 checkpoints to prevent DNA damage from being translated into cell death (Cimprich and Cortez, 2008). Targeting the S/G2 checkpoints is therefore particularly attractive for cancer therapy. Proof-of-principle genetic studies showed that dominant negative inhibition of *ATR* led to abrogation of DNA damage-induced G2 arrest and sensitized cells to a variety of DNA damaging chemotherapeutic agents (Cliby *et al.*, 1998; Nghiem *et al.*, 2001; Caporali *et al.*, 2004; Carrassa *et al.*, 2004; Ward *et al.*, 2004). Despite the attractiveness of the target, small-molecule inhibitors of ATR have proved elusive, and the progress of ATR research has been hampered by the lack of potent inhibitors (Wagner and Kaufmann, 2010). The prototype inhibitor, caffeine (Figure 8), was weak and non-specific but provided sufficiently promising data for the target to be pursued (Sarkaria

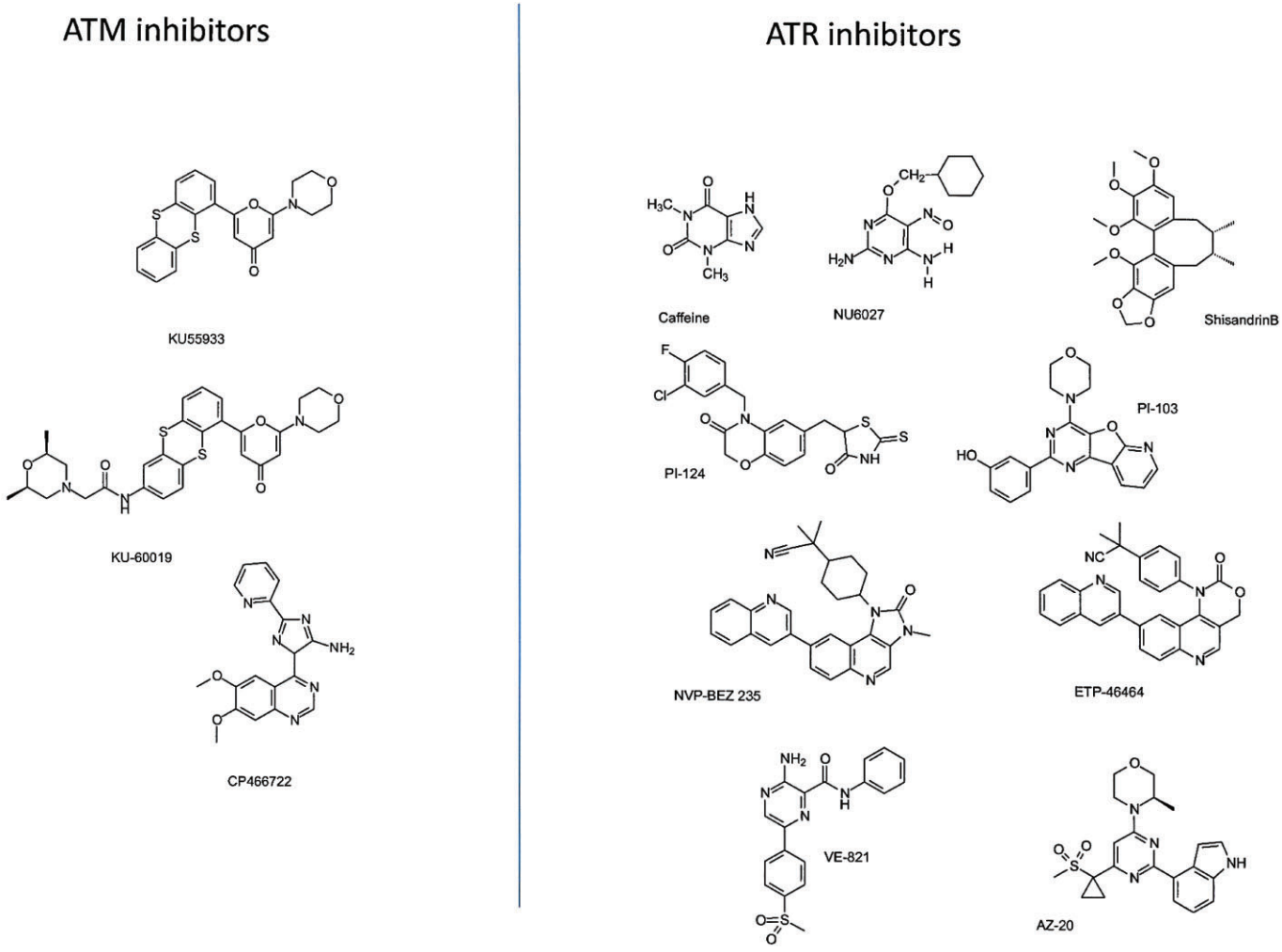

Figure 8
ATM and ATR inhibitors. ATM inhibitors are shown on the left, while ATR inhibitors are shown on the right.

et al., 1999). Schisandrin B, a natural product, was identified as inhibiting ATR, abrogating the UV-induced S and G2/m checkpoint and increasing UV cytotoxicity in human lung cancer cells (Nishida *et al.*, 2009). In a screen of the cross-reactivity of PI3K inhibitors in a panel of PIKKs, PI-103 and PI-124 were identified as being potent ATR inhibitors, with IC_{50} values of 0.9 and 2 μM respectively (Knight *et al.*, 2006). Recently, progress has been made on two fronts. Firstly, the development of a HTS has identified ATR inhibitors (Toledo *et al.*, 2011), including NVP-BEZ235, which had previously thought to be selective for PI3K and mTOR (ATR IC_{50} = 100 nM) and ETP-46464 (ATR IC_{50} = 25 nM). ETP-46464 inhibited the re-start of stalled replication forks and abrogated S-phase arrest after HU exposure (Toledo *et al.*, 2011). Secondly, VE-821, VE-822, NU6027 and AZ-20 have been identified as being ATR inhibitors (Charrier *et al.*, 2011; Peasland *et al.*, 2011; Reaper *et al.*, 2011; Fokas *et al.*, 2012; Jacq *et al.*, 2012) (Figure 8). All drugs inhibited Chk1 phosphorylation at Ser345 and sensitized cells to a variety of DNA damaging agents. VE-821 enhanced IR-induced cytotoxicity in a panel of 12 human cancer cell lines and was more cytotoxic to hypoxic cells (Pires *et al.*, 2012). This compound was more active in cells lacking p53 or ATM function, but chemo- and radiopotentiation by NU6027 was not p53-dependent. AZ-20 was active as a single agent *in vivo*, inhibiting the growth of LoVo xenografts at an p.o. dose of 25 $mg \cdot kg^{-1}$ b.i.d. or 50 $mg \cdot kg^{-1}$ q.d. VE-822 enhanced tumour growth delay induced by X-irradiation and by gemcitabine in xenograft models of human pancreatic cancer (Fokas *et al.*, 2012). NU6027 and VE-822 inhibited RAD51 focus formation, confirming the critical role ATR plays in HRR and was synthetically lethal when BER was genetically inactivated (mutation in XRCC1) or inhibited with a PARPi.

Summary and final conclusions

Targeting the DDR is a viable strategy for cancer therapy, both to reduce resistance to DNA damaging anti-cancer therapy caused by up-regulated repair in cancer cells and also to

specifically target defects in the DDR that render the cancer cells uniquely dependent on retained repair pathways. This latter approach is particularly attractive because (i) it targets the aberration that is likely to have been an early event in the initiation of the cancer (and therefore likely to be present in the majority of the tumour cells and probably in the cancer stem cells) and (ii) it is tumour-specific and unlikely to cause systemic toxicity. Toxicity is a major issue in combination trials of DDR inhibitors with cytotoxic drugs and this has led to the abandonment of MGMT inhibitors. Toxicities have also been observed with PARPi in combination with TMZ and topotecan, which may be due to the assumption that doses of the single agent that are tolerated are appropriate for combination therapy. It is to be hoped that by consideration of the pre-clinical data, and careful titration of both the primary cytotoxic and the PARPi, that combinations which improve the therapeutic index will be identified and PARPi will not suffer the same fate as BG. Radiotherapy may offer the greatest scope for combination with DDR inhibitors.

Inhibitors of other DDR targets – DNA-PK, ATM and ATR – are being investigated, all of which are excellent radiosensitizers in pre-clinical studies. Some of these inhibitors have recently entered clinical trial or are poised to do so in the very near future. The results of these trials are eagerly awaited.

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Conflict of interest

N. J. C. is currently in receipt of research funding from Vertex Pharmaceuticals, Clovis Oncology and BioMarin, has a consultancy agreement with Eisai, and has previously been in receipt of research funding from Agouron, Pfizer Oncology, KuDOS and Astrazeneca, and had a consultancy agreement with Abbott.

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