Chapter 4
Targeting ATR for Cancer Therapy: Profile and Expectations for ATR Inhibitors

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Abstract ATR is a highly versatile player in the DNA damage response (DDR) that signals DNA damage via CHK1 phosphorylation to the S and G2/M cell cycle checkpoints and to promote DNA repair. It is activated by ssDNA, principally occurring due to replication stress that is caused by unrepaired endogenous DNA damage or induced by a variety of anticancer chemotherapy and ionizing radiation. Since an almost ubiquitous feature of cancer cells is loss of G1 control, e.g., through p53 mutation, it is thought that their greater dependence on S and G2/M checkpoint function may render them more susceptible to ATR inhibition. ATR promotes homologous recombination DNA repair and inter-strand cross-link repair. Impairment of ATR function by genetic means or with inhibitors increases the sensitivity of cells to a wide variety of DNA damaging chemotherapy and radiotherapy, with the greatest sensitization observed with gemcitabine and cisplatin. Early inhibitors developed in the 1990s were weak and non-specific but the encouraging data led to the development of more potent and specific inhibitors. We review here the pre-clinical chemo- and radiosensitisation data obtained with these inhibitors that has led to the entry into clinical trial, the potential to combine ATR inhibitors with other DNA repair modulators, and identification of single-agent ATR inhibitor cytotoxicity in cells with activated oncogenes and particular defects in the DDR that may result in greater replication stress or dependence on ATR for survival.

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4.1 Role of ATR in the DNA Damage Response

The DNA damage response (DDR) is a beautifully orchestrated system of DNA damage sensors, transducers and effectors that signal damage for repair and to cell cycle checkpoints to prevent the damage from becoming fixed and transferred to the next generation. Forty years ago DNA was thought to be very stable but Tomas Lindahl found that it was actually being damaged at an astonishing rate, one that would be incompatible with life unless it was also continuously being repaired at a similar rate. In 2015 the Nobel Prize for Chemistry was awarded to Tomas Lindahl, Paul Modrich and Aziz Sancar for their pioneering work on DNA repair (Cleaver 2016). By far and away the most common lesions are single base damage and single strand breaks (SSBs), forming at the rate of 10,000–100,000 lesions per cell/day (Lindahl 1993) and largely due to reactive oxygen species (ROS) resulting from normal metabolism. Whilst these lesions are generally repaired quickly, in replicating cells the rate of repair may not be fast enough to stop the single strand lesions from encountering the advancing replication fork. When this happens the helicase and replication machinery, which are core components of the replication fork, become uncoupled resulting in regions of single stranded DNA (ssDNA). This event has been termed replication stress (RS, recently reviewed in (Taylor and Lindsay 2016)). The impact of this is either stalled replication or the collapse of the fork to reveal a single-ended double strand break (DSB). In addition to unresolved DNA damage from oxidative stress, RS can arise from a number of other events. These include exposure to various exogenous DNA damaging agents such as ultraviolet light (UV), ionizing radiation (IR) and many commonly used cancer chemotherapies. Expression of oncogenes that induce unscheduled proliferation can also lead to RS. This arises when the rate of replication induced by the oncogene is not matched by the metabolic capacity of the cell and the pool of nucleotides required to extend the DNA chain is exhausted. Furthermore, RS can arise from the replication of difficult to copy or fragile regions of the DNA and from highly transcribed regions of DNA, where the transcription and replication machinery can compete for the same region of DNA (Gaillard et al. 2015). Ataxia Telangiectasia Mutated and Rad3-related (ATR) is a vital sensor of RS and a variety of other DNA lesions that generate regions of single-stranded DNA (ssDNA) in double stranded DNA (dsDNA). ATR is critical to cell cycle arrest at the S and G2 checkpoints as well as initiation of DNA repair (Fig. 4.1), as described below. ATR is a member of the PI-3K like family of kinases (PIKKs), which include Ataxia Telangiectasia Mutated (ATM) and DNA-PK_{CS} (DNA-dependent protein kinase catalytic subunit) (Durocher and Jackson 2001), protein kinases that are also involved in the DDR. Many of the phosphorylation substrates of ATR are also common to ATM, and the two are both

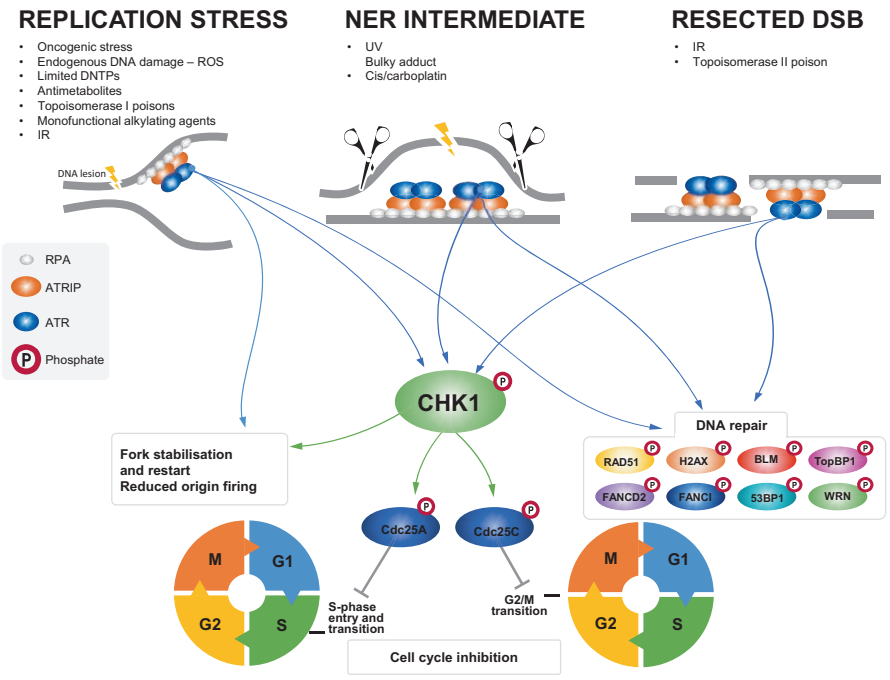


Fig. 4.1 ATR is an apical mediator of the DDR, recruited to regions of ssDNA within dsDNA. The ATR—ATRIP complex is recruited to regions of RPA coated ssDNA within dsDNA that arise at sites of RS and as intermediates during nucleotide excision repair and following resection of DNA DSBs. Once activated, ATR phosphorylates numerous substrates the most important of which is CHK1. This sets off a phosphorylation cascade to coordinate several important cell functions including the arrest of cell cycle by activation of intra-S and G2/M checkpoints, regulation of origin firing, stabilisation of replication forks, and the repair of DNA lesions

involved in the response to DSBs. There is also crosstalk between the two PIKKs. In response to DNA damage ATM and ATR phosphorylate many proteins that are involved in DNA replication, recombination and repair, and cell cycle regulation (Matsuoka et al. 2007).

ssDNA formed at sites of RS is a highly unstable structure that is readily dissected by exonucleases. To prevent this, replication protein A (RPA) is rapidly recruited to ssDNA where it both protects the DNA from exonuclease activity but also acts to recruit proteins that enable the cell to stabilize the stalled fork and repair the damaged DNA. Foremost amongst these is the ATR-interacting protein (ATRIP). ATRIP in-turn recruits ATR (Zou and Elledge 2003; Itakura et al. 2004; Dart et al. 2004). RPA has been shown to activate ATR, and the longer the length of ssDNA the greater the level of ATR activation, supporting the theory that multiple RPA molecules bind the ssDNA to activate ATR (Choi et al. 2010). In the absence of ATR, stalled replication forks collapse resulting in the formation of lethal DSBs and unprotected origins fire, creating increased levels of ssDNA, depleting the RPA pool and ultimately resulting in replication-based catastrophe (Toledo et al. 2013).

The ATR-ATRIP complex is further regulated by a number of other proteins including TopBP1 (Lindsey-Boltz and Sancar 2011), which is recruited to the junction of ssDNA and dsDNA via an interaction with the DNA damage specific RAD9-RAD1-HUS1 clamp (known as 9-1-1). Assembly of this multi-protein complex leads to full activation of the protein kinase activity of ATR.

The activation of ATR is not limited to RS, other cellular events can lead to the generation of ssDNA, which is the catalyst for formation of the ATR protein complex. Such events include generation of intermediates formed during the repair of damaged DNA by the DSB repair, mismatch repair (MMR) and nucleotide excision repair (NER) pathways. DSBs, such as those caused by IR may be repaired by any one of four pathways: Non-homologous end joining (NHEJ), alternative NHEJ, homologous recombination repair (HRR) or single-strand annealing (reviewed in (Mlasenov et al. 2016)). Of these the only pathway that preserves the DNA sequence as well as restoring DNA integrity is HRR, with which ATR is intimately connected, as it uses the sister chromatid as a template for repair. An obligatory early step in HRR is the processing of the DNA DSB by end resection to generate a 3′ overhang. End resection is achieved by a number of steps: CtIP, which is an ATR phosphorylation target, recruits the MRN complex (composed of Mre11, Rad50 and Nbs1). MRE11 has 3′-5′ exonuclease activity and initiates end resection, which is continued by EXO 1 following its recruitment by CtIP. The DNA2/BLM complex then unwinds the DNA and the DNA2 component completes end resection. RPA coats the ssDNA to recruit ATRIP and ATR (Fig. 4.1) (Shiotani and Zou 2009; Symington and Gautier 2011).

MMR acts on DNA lesions caused by insertion or deletion loops resulting from replication errors and mismatches in the DNA base-pairs that often occur due to alkylating mutagens (Fang et al. 1993). One of the primary events during MMR is formation of Mut protein complexes (MutSα, Sβ and MutLα) and nuclease excision of the aberrant bases. This reveals ssDNA that is subsequently coated by RPA (Genschel and Modrich 2009), leading to the recruitment of ATR. It is thought that Mut complexes act as scaffolds for proteins involved in DNA repair and checkpoint activation, such as ATR (Liu et al. 2010). In keeping with this, depletion of MSH2 (a component of the MutSα complex) by siRNA blocks ATR activity (Wang and Qin 2003); MMR-proficient cells form ATR foci following DNA alkylation, in contrast to MMR-deficient cells (Caporali et al. 2004); and MSH3 (a component of the MutSβ complex) binds hairpin loops in RPA-coated ssDNA to recruit ATRIP and activate ATR (Burdova et al. 2015). Furthermore, the G2 arrest associated with the repair of the mismatch repair (MMR) substrate, 6-thioguanine, has been shown to be ATR dependent (Yamane et al. 2004). Another common source of DNA damage is UV and environmental chemicals that form DNA distorting adducts, these are repaired by nucleotide excision repair (NER). At sites of damage a number of protein complexes are formed that include the DNA-damage binding (DDB) complexes DDB1 and DDB2 and the XPC-Rad23B complex. These in turn recruit exonuclease activities such as ERCC1 that excise 20–30 nucleotides of DNA around the damage site on one of the strands of DNA, leaving a portion of ssDNA as a template for repair by polymerase activities (Sibghatullah et al. 1989).

Once activated at regions of RPA coated ssDNA, ATR can phosphorylate a very long list of putative substrates and although the molecular consequences for many of these are yet to be determined it is clear that they control a number of important cellular functions that include promotion of cell survival, arrest of replication and cell cycle, stabilisation of the stalled fork and repair of the damaged DNA (Myers et al. 2009). These events are described in more detail below.

4.1.1 ATR Signaling to Regulate DNA Replication and Cell Cycle Progression

The best characterized substrate for ATR is the checkpoint kinase CHK1. ATR mediates CHK1 activation by phosphorylation at residues S317 and S345, both of which are required for CHK1 activation (Zhao and Piwnica-Worms 2001). Phosphorylation of CHK1^{ser345} by ATR is often used as a marker of ATR activity (Peasland et al. 2011; Liu et al. 2000; Parsels et al. 2011). Upon phosphorylation at both these residues CHK1 becomes active, triggering autophosphorylation at serine 296 (Parsels et al. 2011) and phosphorylation of a number of downstream targets involved in DNA repair and cell cycle arrest. CHK1 controls entry into mitosis via the G2/M checkpoint, and S-phase progression via the intra S-phase checkpoint. Following RS, ATR mediated activation of CHK1 leads to the phosphorylation of the CDC25 phosphatase proteins CDC25A and B. This results in the inhibition of phosphatase activity, which in turn leads to the persistence of an inhibitory phosphorylation event on CDK1. In addition, CHK1 phosphorylates and activates the Wee1 kinase, which directly induces the inhibitory phosphorylation on CDK1 (Fig. 4.1) (Sorensen and Syljuasen 2012; Chen et al. 2003; Dai and Grant 2010; Lee et al. 2001). The outcome of this cascade is to block CDK1-mediated mitotic progression. In doing so the ATR-CHK1 response prevents cells with damaged chromosomes from entering mitosis, which could otherwise lead to gross genetic deformations or mitotic catastrophe. In addition to a role in preventing entry into mitosis, recent evidence suggests that ATR, via CHK1 activation, can also impact the progression of cells with damaged DNA through mitosis. ATR-CHK1 activation leads to phosphorylation of Aurora-B, a kinase that is involved in the mitotic spindle checkpoint. This checkpoint serves to ensure that duplicated chromosomes are correctly segregated to opposing cell poles. Accordingly, defective chromosomes that arise from persistent DNA damage or aberrant repair are not segregated and progressed through to cytokinesis as a result of CHK1 mediated Aurora-B activation (Mackay and Ullman 2015).

ATR-mediated phosphorylation of CHK1 and its downstream effects on CDC25 protein stability are also a key event in the regulation of the intra-S-phase checkpoint. Cdc25A removes an inactivating phosphorylation on the CDK2/Cyclin A or E complexes, which promotes S-phase. Following exposure to a variety of DNA damaging agents, including IR (Sorensen et al. 2003), UV and hydroxurea (HU)

(Mailand et al. 2000; Xiao et al. 2003), CHK1 promotes the rapid degradation of Cdc25A, preventing S-phase entry (Dai and Grant 2010). Accordingly, ATR knockdown has been shown to stabilise Cdc25A (Sorensen et al. 2004). In addition to a role in regulating cell cycle checkpoints, ATR-mediated phosphorylation of CHK1 can also suppress global firing of new replication origins, via the CDC25-mediated suppression of CDK activity. This acts to stop DNA replication, avoiding the potential to form multiple unstable forks and catastrophic DNA damage. Interestingly, recent studies have shown that ATR activity can also suppress replication origin firing via a CHK1 independent route that involves the helicase SMARCAL1 (Couch et al. 2013). The effect of ATR-mediated CHK1 activation on DNA replication and cell cycle progression is therefore fourfold: phosphorylation of CDC25 is inhibitory, thereby preventing S phase entry and preventing G2/M transition; Wee1 is phosphorylated and stabilised resulting in inactivation of CDK1, Aurora-B is activated blocking G2/M transition; and CDC25 mediated suppression of origin firing.

The impact of ATR on DNA replication and S and G2/M cell cycle progression following DNA damage is important when considering the potential benefit from inhibiting ATR. Specifically, impairment of G1 control in cancer is almost ubiquitous (Massague 2004), caused by multiple mechanisms such as loss of function of key G1 control proteins including p53 or Rb. For example, the *TP53* gene is the most commonly mutated gene in cancer with >50% of all solid tumours harboring mutations largely in the DNA binding domain of the *TP53* gene (Olivier et al. 2010). This sets up an hypothesis that cancer cells defective in the G1 checkpoint may be reliant on the ATR mediated S and G2/M checkpoints for survival from DNA damage. In contrast, non-cancer cells with their full complement of cell cycle checkpoints may better tolerate ATR inhibition. The most convincing data regarding G1 dependence comes from experiments using paired isogenic cell lines that differ only in their p53 status (discussed in more detail below). For example, ATR depletion sensitised human colorectal cells with inactive p53 to cisplatin but when wt p53 was knocked in survival was increased (Sangster-Guity et al. 2011). However, it is important to acknowledge that other studies have shown p53 competent cancer cells can also be sensitive to ATR inhibitors. This may be associated with other defects in the G1 checkpoint. For example, ATR silencing sensitized p53 wild-type U2OS cells, which have G1 dysfunction by virtue of p16 deletion, to topoisomerase I poisons (Flatten et al. 2005). Moreover, U2OS sensitivity to dominant negative inactivation of ATR was further enhanced by inducing additional defects in G1 control (Nghiem et al. 2001).

4.1.2 ATR Signaling to DNA Repair

Once DNA damage is detected, the cell can employ a series of distinct repair processes. This is determined by a number of factors most notable of which is the nature of the damage lesion e.g., small vs. bulky adducts, single strand vs. double strand breaks; and the phase of cell cycle in which the damage is detected. During

the S- and G2 phases of cell cycle the cell can use the sister chromatid DNA as a template for repair, enabling high fidelity repair by HRR. In contrast, outside of these cell cycle phases, multiple repair pathways are available. Lesions affecting one strand of DNA can be repaired with high fidelity using the complementary strand as a template but for lesions affecting both strands there is a considerable risk of incorrect repair, resulting in genome instability. HRR involves three major steps; end-resection (as described above) of a DSB to reveal a region of ssDNA (redundant in the case of a stalled fork), invasion of the DNA into the sister chromatid and then finally damage resolution, a process which includes extension of the DNA chain by DNA polymerase activity and reannealing of the DNA ends. Although it is far from clear exactly how ATR affects DNA damage repair, multiple strands of evidence implicate ATR in the regulation of HRR. Firstly, a number of studies have shown that depletion of ATR leads to a decrease in the efficiency or frequency of HRR (Wang et al. 2004; Brown et al. 2014). Secondly, ATR expression is cell cycle dependent peaking at S and G2, coincident with HRR: ATR is associated with chromatin throughout the cell cycle in the absence of genotoxic stress, however this degree of association appears to be much higher in S phase when the threat to genomic integrity is the greatest (Dart et al. 2004). Thirdly, inhibition of ATR has been shown to decrease a number of HRR markers most notable amongst which is the RAD51 filament protein that is involved in the homology search. Fourthly, ATR is associated with, or phosphorylates a number of proteins that are involved in HRR. These include the RecQ helicases BLM and WRN (Blm suppresses inappropriate sister chromatid exchange and WRN prevents the collapse of stalled forks); the breast cancer type 1 susceptibility protein (BRCA, which is involved with the recruitment of HRR essential repair proteins); and a minor variant of histone H2A (H2AX, which colocalises with a series of HRR proteins including BRCA1 and RAD51). Finally, TopBP1, an important regulator of the ATR protein complex, has been shown to interact with NBS1. This protein forms part of the MRN complex that plays an important role in activating HRR (Morishima et al. 2007).

At sites of RS, in the event that ATR fails to stabilize the replication fork and repair the causative lesion, the replication fork can rapidly collapse to form a potentially lethal DSB. Once a DSB is formed a surveillance pathway, mediated by the ATR homolog ATM, determines the fate of the cell. ATM signals to cell cycle arrest via phosphorylation of critical downstream substrates such as p53 and CHK2, and also triggers repair of the DSB by HRR. Notably, it has been widely reported that components of the ATM DSB pathway are very commonly dysfunctional in cancer. Most notable examples include loss of the ATM activating complex MRN, which has been observed in breast cancer (Bartkova et al. 2008; Jiang et al. 2009); loss of function mutations or loss of expression of ATM itself, which has been observed in a number of cancers such as non-small cell lung cancer (Weber et al. 2016); and perhaps most importantly, loss of function mutations in the key ATM substrate, p53. This defect is highly prevalent in some aggressive diseases such as serous ovarian cancer (>95%) (The Cancer Genome Atlas Research Network 2011; Cole et al. 2016), basal-like breast cancer (80%) (Cancer Genome Atlas Network 2012a) and squamous cell lung cancers (>80%) (Cancer Genome Atlas Network 2012b).

Defective DSB repair associated with defects in ATM signaling has been suggested to be an early event in tumourgenesis and provides the nascent tumour with an environment that supports genomic instability, as a result of persistent unrepaired DNA damage. Although a defective ATM-p53 response may provide a growth advantage to the tumour it increases the reliance on the ATR RS response to survive DNA damage during replication (Halazonetis et al. 2008).

In addition to a role in HRR, ATR has also been implicated in the regulation of the interstrand crosslink (ICL) repair pathway. This complex pathway acts to resolve adducts formed between two complementary DNA strands, and utilizes numerous repair processes. Typically, such adducts arise from treatment with anti-cancer drugs such as the bifunctional N-mustards, platinating agents like cisplatin and carboplatin; and mitomycin C. During replication, at the site of an ICL, a large protein complex is formed—known as the Fanconi Anemia (FA) core. This complex consists of over ten separate proteins and although the full activity of this complex is not yet defined it is clear that one effect is to recruit endonuclease activities (such as the XPF-ERCC1 or FAN1 endonucleases) that cleave either side of the ICL to unhook the adduct from one of the DNA strands. The result is a DSB that is repaired by a combination of translesion synthesis (to fill the gap left by the excised adduct, restoring the growing DNA chain) and HRR. The remaining 'still hooked' strand, which now constitutes a bulky adduct, is resolved by NER. A comprehensive description of ICL has been reviewed elsewhere (Kim and D'Andrea 2012; Haynes et al. 2015; Deans and West 2011). ATR has been associated with ICL through a series of important experimental observations. ATR phosphorylates and regulates many FA proteins. Specifically, ATR has been shown to phosphorylate FANCs A, G, E, I, M and D2. In many cases it has been shown that ATR-mediated phosphorylation directly impacts their function, for example, blocking the ATR site of phosphorylation in FANCM impacts its recruitment to the sites of ICL (Singh et al. 2013). In addition to a role for ATR in the regulation of FA core proteins, the converse has also been shown: FA core proteins can lead to activation of ATR. In response to damage, the FA protein FANCM has been shown to activate ATR and its downstream intra S-phase checkpoint. Additionally, knockdown of *FANCM* or *FAAP24* (a gene that encodes the FA core-complex associated protein 24) reduced ATR-dependent phosphorylation of pCHK1^{Ser317} and p53^{Ser15} following HU and UV, respectively, (Collis et al. 2008); and deletion of *FANCM* reduced levels of the ATR marker pCHK1^{Ser345} following treatment with camptothecin (Schwab et al. 2010). Furthermore, depletion of FANCM in cells leads to a phenotype that is very similar to that seen with loss of ATR: increased DNA damage and cell cycle checkpoint defects in response to RS (Luke-Glaser et al. 2010). Finally, the FA core complex has been reported to increase the binding of ATRIP to chromatin at sites of damage, which in turn leads to activation of ATR (Tomida et al. 2013).

In addition to the potentially beneficial anti-cancer impact inhibiting ATR has on cancer cell cycle control, blocking its impact on DNA damage repair may also provide substantial benefit. This is based on a common finding that many cancer cells have defects in overlapping repair pathways, which in turn may place a burden on

remaining repair capacity. The most notable example is a defect in the ATM-p53 mediated DSB response pathway that is described above. In the absence of a functional ATM-p53 response, cells may be especially reliant on ATR to maintain survival in the face of RS. This is discussed in detail later in this chapter. Additionally, defects in other repair pathways may lead to an increase in RS from persistence of unrepaired DNA damage as the cell progresses to S-phase of the cell cycle. In turn this would increase the requirement for ATR activity to maintain cell survival.

The remainder of this chapter is dedicated to reviewing the data that supports the potential for ATR inhibitors to be used in a variety of contexts to provide patient benefit.

4.2 Validation of ATR as a Therapeutic Target

Genomic instability has been identified as an "enabling characteristic" of cancer cells (Hanahan and Weinberg 2011), and commonly arises due to errors in DNA replication and repair machinery. Defects in the DDR are common in cancer, leading to a reduced repair capacity in many cancer cells compared with normal cells. Historically, conventional cytotoxics that act by damaging DNA have relied on exploitation of these defects. Many of the anticancer drugs in routine clinical use act with the intention of inducing lethal levels of DNA damage in the tumour. These drugs can be classified based on the form of DNA damage they induce: single base damage through alkyltation for example by temozolomide or dacarbazine (DTIC); single strand breaks induced by topoisomerase I inhibitors such as irinotecan, topotecan and camptothecin; DSBs induced by topoisomerase II inhibitors such as etoposide, doxorubicin and mitoxantrone; DNA cross-links (inter-or intra strands) induced by bifunctional alkylating agents such as the nitrogen mustards, such as melphalan, and the platinum drugs cisplatin and carboplatin; and finally, the antimetabolite class of drugs typified by the nucleotide analogs gemcitabine, HU and 5-fluoro uracil (5-FU) and antifolates such as methotrexate and pemetrexed that induce DNA damage both by blocking DNA extension via insertion in the extending chain and by inhibiting the synthesis of the deoxynucleotides that are essential for DNA replication (the lack of which will lead to RS). For many patients treatment with these DNA damaging drugs provides limited benefit, and several strands of clinical evidence suggest that functional capacity of the DNA damage response network is an important determinant in response. For example, several studies have been reported where good responses to such drugs are associated with impaired DNA repair processes. Firstly, cisplatin based treatment leads to a remarkable cure rate of >80% in patients with testicular cancer (Masters and Koberle 2003). Cell studies have demonstrated that platinum adducts in testicular cancer cells persist, presumably as a result of failed repair, in contrast to observations with cells from other tumour types. Low levels of key proteins involved in NER, such as ERCC1-XPF and XPA in testicular cancer cells may suggest that defective NER is a driver

of the cancer cell sensitivity to cisplatin (Masters and Koberle 2003). Secondly, a recently reported clinical study assessed the response of patients with triple negative breast cancer to carboplatin. The investigators noted that patients with a germline BRCA mutation (a protein that is involved in HR repair) responded better to carboplatin than the BRCA wild type group (68% vs. 28% overall response rate respectively) (Tutt et al. 2015).

Since so many of these agents lead to activation of ATR, it has long been considered a suitable target for anticancer therapy. Importantly, a number of observations in mouse and humans provide confidence that modulation of ATR activity could be tolerated by non-cancer cells. Although ATR knockout mice are not viable (Brown and Baltimore 2000) and significant depletion of ATR in mice and humans leads to developmental issues (Seckel syndrome in humans, which is associated with microcephaly and short stature) (O'Driscoll et al. 2004), conditional knockout in adult mice is well tolerated and no enhanced cancer risk is observed in either Seckel patients or conditional knockout mice (Ruzankina et al. 2007; Schoppy et al. 2012; Murga et al. 2009).

Despite the potential for drugging ATR, the lack of suitable inhibitors meant that initially genetic manipulation was the only means of target validation (Table 4.1). Using a human transformed fibroblast cell line (GM847) or an osteosacrcoma line (U2OS) expressing a doxycycline-inducible ATR-kinase dead gene, that acts in a dominant negative fashion (Nghiem et al. 2001, 2002; Cliby et al. 1998, 2002), it was shown that ATR inactivation sensitised cells to the monofunctional alkylating agent, methylmethanosulfonate (MMS), the cross-linking agent cisplatin, the topoisomerase I and II inhibitors topotecan, SN38 (the cell active metabolite of irinotecan), the topoisomerase II poisons, doxorubicin, etoposide and teniposide; IR, and HU but not to taxanes, which exert their antiproliferative effect largely through inhibiting mictotubule dynamics necessary for chromosomal segregation at mitosis (Cliby et al. 1998). A second approach assessed the impact of Seckel mutant ATR expression, which leads to low ATR activity, on the sensitivity of DLD1 cancer cells to a range of drugs and irradiation (Hurley et al. 2007). These cells were six-fold more sensitive to the topoisomerase II poison doxorubicin, 10–20-fold more sensitive to the antimetabolites 5-fluorouracil, gemcitabine, HU and methotrexate and >400-fold more sensitive to cisplatin than DLD1 cells expressing wild-type ATR (Wilsker and Bunz 2007). A third approach, adopted by a number of investigators, used siRNA knockdown of ATR. In a range of cancer cell backgrounds, ATR knockdown led to increased sensitivity, when compared with control siRNA treated cells, to cisplatin, MMS, temozolomide, topotecan, SN38, and the antimetabolite gemcitabine. (Caporali et al. 2004, 2008; Wagner and Karnitz 2009; Huntoon et al. 2013). Taken together these data supported the hypothesis that inhibiting ATR could be an attractive approach to treating cancer, and specifically to improve the benefit from the DNA damaging drugs that are widely used as standard of care across many indications.

Table 4.1 Summary of sensitisation to DNA damaging agents by genetic ATR inactivation

Treatment class	Treatment	Cell line/genetic inactivation	Increased sensitivity?	Reference
X-irradiation	IR	GM847-KD	Yes	Cliby et al. (1998)
		GM637-KD	Yes	Wright et al. (1998)
		U2OS-KD	Yes	Nghiem et al. (2002)
		F02-98 (Seckel patient fibroblasts)	Slight	O'Driscoll et al. (2003)
		DLD1-ATR-Seckel	Yes	Hurley et al. (2007)
UV irradiation	UV	GM847-KD	Slight	Cliby et al. (1998)
		GM637-KD	Yes	Wright et al. (1998)
		U2OS-KD	Yes	Nghiem et al. (2002)
		F02-98 (Seckel patient fibroblasts)	Yes	Yang et al. (2008)
		DLD1-ATR-Seckel	Yes	Hurley et al. (2007)
Alkylating agents	Cisplatin	GM847-KD	Yes	Cliby et al. (1998)
		U2OS-KD	Yes	Nghiem et al. (2002)
		DLD1-ATR-Seckel	Yes	Wilsker and Bunz (2007); Wilsker et al. (2012)
		HeLa siRNA	Yes	Wagner and Karnitz (2009)
		U2OS siRNA	Yes	Wagner and Karnitz (2009)
		HCT116 siRNA	Yes	Wagner and Karnitz (2009)
		OVCAR-8 siRNA	Yes	Wagner and Karnitz (2009)
	Cyclophosphamide	DLD1-ATR-Seckel	Yes	Wilsker and Bunz (2007)
	Mitomycin C (MMC)	F02-98 (Seckel patient fibroblasts)	Yes	Yang et al. (2008)
		DLD1-ATR-Seckel	Yes	Wilsker and Bunz (2007); Wilsker et al. (2012)
	Methyl methanosulfonate	GM847-KD	Yes	Cliby et al. (1998)
		PC3 siRNA	Yes	Collis et al. (2003)
		DU145 siRNA	Yes	Collis et al. (2003)
	Temozolomide	DK0064 (ATR mutant) lymphoblastoid, LN229 glioma and DO3 melanoma siATR	Yes	Eich et al. (2013)

(continued)

Table 4.1 (continued)

Treatment class	Treatment	Cell line/genetic inactivation	Increased sensitivity?	Reference
Topoisomerase I poisons/inhibitors	Topotecan	GM847-KD	Yes	Cliby et al. (2002)
		OVCAR-8 siRNA	Yes	Huntoon et al. (2013)
	SN-38 (irinotecan)	GM847-KD	Yes	Cliby et al. (2002)
		HeLa siRNA	Yes	Flatten et al. (2005)
		U2OS siRNA	Yes	Flatten et al. (2005)
	camptothecin	HeLa siRNA	Yes	Flatten et al. (2005)
Topoisomerase II poisons/inhibitors	Etoposide	GM847-KD	Yes	Cliby et al. (2002)
	Teniposide	DLD1-ATR-Seckel	Yes	Wilsker et al. (2012)
	Doxorubicin	GM847-KD	Yes	Cliby et al. (2002)
		DLD1-ATR-Seckel	Yes	Wilsker and Bunz (2007)
Antimetabolites	5-FU	DLD1-ATR-Seckel	Yes	Wilsker and Bunz (2007)
	Gemcitabine	DLD1-ATR-Seckel	Yes	Wilsker and Bunz (2007)
		HeLa siRNA	Yes	Wagner and Karnitz (2009)
		U2OS siRNA	Yes	Wagner and Karnitz (2009)
		HCT116 siRNA	Yes	Wagner and Karnitz (2009)
		OVCAR-8 siRNA	Yes	Huntoon et al. (2013)
	HU	GM847-KD	Yes	Cliby et al. (1998); Peasland et al. (2011)
		U2OS-KD	Yes	Nghiem et al. (2002)
		DLD1-ATR-Seckel	Yes	Wilsker and Bunz (2007)
	Methotrexate	DLD1-ATR-Seckel	Yes	Wilsker and Bunz (2007)
	Raltitrexed	DLD1-ATR-Seckel	Yes	Wilsker and Bunz (2007)
PARP inhibitor	Rucaparib	GM847-KD	Yes	Peasland et al. (2011)
	Veliparib	OVCAR-8 siRNA	Yes	Huntoon et al. (2013)
Taxanes	Paclitaxel	GM847-KD	No	Cliby et al. (2002)
		DLD1-ATR-Seckel	No	Wilsker and Bunz (2007)

4.3 Development of ATR Inhibitors

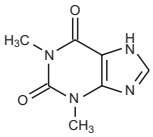
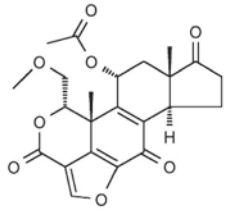
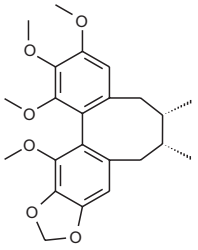
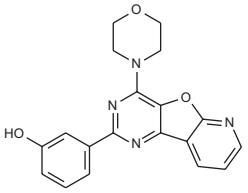
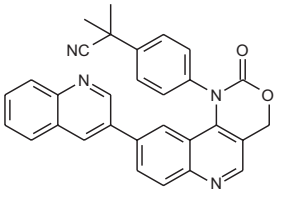
For many years the available ATR inhibitors lacked potency and specificity but nevertheless were used as tools to support data generated from genetic studies (Table 4.2). One of the earliest ATR inhibitors was caffeine, but it lacked potency (IC_{50} = 1.1 mM) (Cortez 2003) and specificity, as it is a more potent inhibitor of ATM (IC_{50} = 0.2 mM) (Sarkaria et al. 1999). Wortmannin, a fungal metabolite, is a more potent inhibitor of ATR than caffeine (IC_{50} = 1.8 μM) but inhibits multiple PIKKs including ATM (IC_{50} = 150 nM). PI-103, a PI3K inhibitor also inhibits ATR (IC_{50} = 850 nM) but had equivalent potency against ATM (IC_{50} = 920 nM) and greater potency against DNA-PK (IC_{50} = 2 nM) (Knight et al. 2006). The natural product, Schisandrin B is a weak inhibitor of ATR with an in vitro IC_{50} of 7.25 μM, however at concentrations of 30 μM or greater, no inhibition of related kinases was observed (ATM, CHK1, PI3K, DNA-PK and mTOR), indicating potential for ATR selectivity (Nishida et al. 2009). NU6027 (2,6-diamino-4-cyclohexyl-methyloxy-5-nitroso-pyrimidine), originally designed as a Cdk2 inhibitor, was found to be more efficient in inhibiting cellular ATR activity (as determined by CHK1 serine^{345} phosphorylation) than CDK2 activity (IC_{50} = 6.7 μM for ATR and >10 μM for Cdk2), with no effect on ATM and DNA-PK (Peasland et al. 2011). Finally, a high-throughput screen of 623 compounds identified that the PI-3K inhibitor ETP-46464 was a potent ATR inhibitor (IC_{50} = 25 nM) (Toledo et al. 2011; Teng et al. 2015).

The discovery of more potent and specific inhibitors of ATR may have been hampered by challenges in accessing the protein to run high throughput screens and to support medicinal chemistry efforts (ATR is a very large protein (Unsal-Kacmaz and Sancar 2004)). However, in recent years a number of potent and specific ATR inhibitors have been reported (Table 4.1 and reviewed in (Foote et al. 2015)) aided by successful production of recombinant protein, elegant cell screens and optimization of compounds initially designed to inhibit close analogs of ATR such as mTOR. Of these compounds VE-821 and VE-822 from Vertex Pharmaceuticals (recently licensed to Merck, EMD Serono); and AZ20 and AZD6738 from AstraZeneca, have been most widely used in pre-clinical studies (Foote et al. 2013; Guichard et al. 2013; Jones et al. 2013; Reaper et al. 2011; Charrier et al. 2011). VE-822 (also known as VX-970 and more recently as M6620), an analog VX-803 (M4344) and AZD6738 have all progressed in to clinical development (discussed in detail in Chap. 5 of this volume). These advanced compounds have greatly expanded the tool box available to researchers.

4.4 ATR Inhibition as Combination Therapy with DNA Damaging Chemotherapy

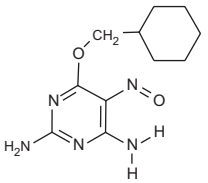
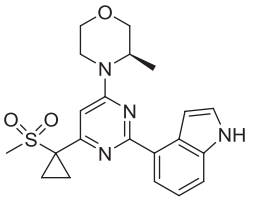
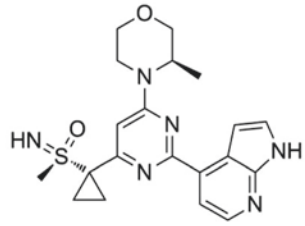
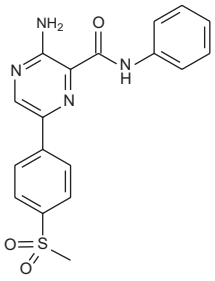
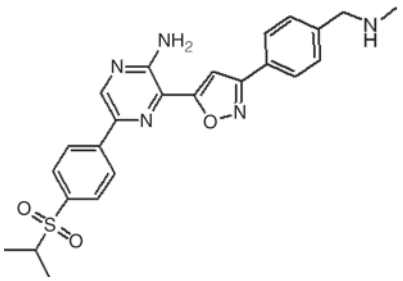
Since the advent of potent and specific ATR inhibitors, more detailed assessments have been possible that have provided insights on the impact ATR inhibition has on non-cancer cells, which cancer cells are most susceptible to ATR inhibition and finally the in vivo profile of ATR inhibition in mouse models of cancer. Early studies

Table 4.2 ATR inhibitors

Compound and structure	Ki or IC_{50} for ATR	Other reported targets
 Caffeine	IC_{50} = 1.1 mM	ATM, DNA-PKcs mTOR
 Wortmanin	IC_{50} = 1.8 μM	ATM, DNA-PKcs, PI3K
 Schisandrin B	IC_{50} = 7.25 μM	
 PI-103	IC_{50} = 0.9 μM	PI3K, mTOR, DNA-PKcs
 ETP-46464	IC_{50} = 25 nM	

(continued)

Table 4.2 (continued)

Compound and structure	Ki or IC_{50} for ATR	Other reported targets
 NU6027	Ki = 100 nM Cellular IC_{50} = 6.7 μM	CDK2
 AZ20	IC_{50} = 4.5 nM	
 AZD6738	IC_{50} = 1.0 nM	
 VE-821	IC_{50} = 13 nM	
 VX-970 (M6620)	IC_{50} = 0.2 nM	

with NU6027 revealed that, at concentrations that were not cytotoxic per se, NU6027 sensitised MCF7 breast cancer cells to IR, temozolomide, cisplatin, camptothecin, doxorubicin, and hydroxyurea, but not the anti-tubulin agent paclitaxel (Peasland et al. 2011). Similarly ETP-46464 enhanced the radiation and cisplatin sensitivity of human ovarian, endometrial and cervical cancer cell lines (Teng et al. 2015). Using VE-821 it was shown that ATR inhibition markedly sensitised HCT116 colon cancer cells to gemcitabine, camptothecin, etoposide, carboplatin and cisplatin (addition of VE-821 decreased the IC_{50} value for the DNA damaging drug by up to ten-fold) (Reaper et al. 2011). As expected, no sensitization was observed with taxotere. A number of subsequent experiments have confirmed the strong potentiation of DNA damaging drug induced cell death by ATR inhibition in cancer cells. For example, in a panel of cancer and non-cancer cell lines VE-821 sensitised most of the 14 cancer lines to cisplatin, in stark contrast, potentiation of cisplatin was not observed for any of the six non-cancer cell lines (Reaper et al. 2011). The apparent cancer specific activity of VE-821 was further characterized in H23 cancer cells and HFl1 non-cancer fibroblast cells. In both cell lines at early time points (24 h), VE-821 enhanced the cytostatic activity of cisplatin. By 96 h this had translated to marked potentiation of cell death in the cancer cell line in contrast to the non-cancer cells in which no enhancement of cell death was observed (Reaper et al. 2011). Furthermore, the enhanced growth arrest that was observed in the non-cancer cells was reversed when VE-821 and cisplatin were washed off. Tolerance of the non-cancer cells to treatment of VE-821 with cisplatin was shown by western blot to be associated with a rapid activation of ATM leading to a compensatory DDR involving activation of a number of downstream ATM cell cycle checkpoint proteins such as CHK2 (Reaper et al. 2011). In a complementary experiment using matched or isogenic cell pairs it was shown that loss of p53 by siRNA depletion or expression of the E6 papillomavirus was sufficient to sensitise cells to co-treatment with VE-821 and cisplatin (Reaper et al. 2011). Similarly, loss of expression or inhibition of ATM sensitized cisplatin treated non-cancer cells to ATR inhibition by VE-821 (Reaper et al. 2011). However, a number of studies have shown individual cell lines can respond to ATR inhibition despite being wild type for p53 (Peasland et al. 2011; Hall et al. 2014). This may be due to defects elsewhere in the ATM-p53 pathway or it may suggest that some cells can be reliant on ATR activity under high RS pressure even in the presence of a fully functioning ATM-p53 response. Additional studies to better define markers of response that can be used to identify target patient sub-populations are merited.

In vivo benefit from ATR inhibition in combination with DNA damaging drugs has been demonstrated in a number of separate studies using both human cancer cell line and primary patient derived tumour xenografts. Marked anti-cancer activity associated with substantial synergy has been demonstrated in combination with cisplatin, gemcitabine and irinotecan (Hall et al. 2014; Jossé et al. 2014; Vendetti et al. 2015; Pollard et al. 2016a). As an example, in one study a panel of seven patient derived non-small cell lung cancer xenografts were treated with cisplatin or VX-970

(M6620) alone or with the combination. The ATR inhibitor had no impact on tumour growth alone in any of the models, whereas cisplatin gave a range of responses: three tumours responded well with >70% tumour growth inhibition, one showed a moderate response (50–70% tumour growth inhibition), and three were insensitive (<20% tumour growth inhibition). In six of the seven models the combination was statistically more effective than cisplatin alone. Notably, complete tumour growth inhibition was observed in all three of the cisplatin resistant models and complete tumour regression was observed in one cisplatin responsive model (Hall et al. 2014). This raises the attractive prospect that ATR inhibition may be an approach to resensitise platinum resistant tumours as well as increasing the benefit in cisplatin responsive tumours. In this study, activity was associated with inhibition of CHK1 phosphorylation in tumours and the combination of VX-970 (M6620) and cisplatin was well tolerated. A similarly beneficial profile in combination with cisplatin has been demonstrated with AZD6738 in an H23 lung cancer xenograft model (Vendetti et al. 2015). Robust anti-tumour activity with well-tolerated ATR and DNA damaging drug combinations is consistent with in vitro observations that ATR inhibition leads to cancer cell specific enhancement of cell death. A second important observation was made from a study of irinotecan and VX-970 (M6620), where it was shown that the combination of an ATR inhibitor with a DNA damaging drug was capable of providing greater efficacy than could be obtained with the DNA damaging drug alone at its maximum tolerated dose (MTD) (Jossé et al. 2014).

A key consideration when developing agents with a novel mechanism of action is dose schedule, this is even more relevant when studies involve drug combinations. The first systematic pre-clinical analysis of dose schedule for combinations of ATR inhibitors with DNA damaging drugs was reported at the 2016 Annual AACR meeting (Pollard et al. 2016a). Maximum in vitro and in vivo activity for VX-970 (M6620) in combination with cisplatin or gemcitabine was achieved when the ATR inhibitor was administered after the DNA damaging therapy. Cell studies demonstrated that transient exposure to ATR inhibition for just 2 h was sufficient for response, with optimal activity when addition of the ATR inhibitor was timed to coincide with peak accumulation of cells in S-phase and concomitant activation of ATR (P-CHK1), following treatment with the DNA damaging drug. In mouse models the optimal schedule was VX-970 (M6620) administered 12–24 h after chemotherapy (Pollard et al. 2016a).

Given the marked ability of ATR inhibitors to potentiate the anti-cancer activity of DNA damaging drugs, with minimal impact on non-cancer cell viability, plus the prevalence of these drugs as standard-of-care across most cancer indications, ATR inhibitors represent an exciting novel therapeutic approach. Accordingly, a number of clinical studies are ongoing with AZ6738, VX-970 (M6620) and VX-803 (M4344) in combination with the DNA damaging drugs cisplatin, carboplatin, gemcitabine and topotecan in a range of cancer indications (Reviewed in Chap. 5).

4.5 ATR Inhibition as Combination Therapy with Ionising Radiation (IR)

IR is used to treat about 60% of cancer patients, both as a potentially curative therapy and also to palliate symptoms. Furthermore, IR is one of the most successful curative therapies used in cancer treatment with about 40% of cancer cures involving IR treatment (Ringborg et al. 2003). Cell death from IR is associated with lethal DNA damage arising both from the direct interaction of radiation with the DNA or, more commonly, indirectly via the ionization of water or oxygen molecules to form highly reactive species within the vicinity of the DNA (Lomax et al. 2013). DNA damage from IR treatment includes single stranded breaks, RS and double strand breaks (Lomax et al. 2013). A number of early studies, involving the expression of inactive ATR mutants demonstrated an important role for ATR and downstream HRR in the response to, and repair of, IR mediated DNA damage (Wang et al. 2004; Cliby et al. 1998; Wright et al. 1998). Potentiation of IR by inhibition of ATR was first demonstrated in vitro with the semi-selective inhibitor NU6027 in MCF7 breast cancer cells. In this experiment the ATR inhibitor decreased clonogenic cancer cell survival by >80% in combination with IR, compared with ~50% survival for IR alone (Peasland et al. 2011). A comprehensive assessment of the benefit from ATR inhibition with IR was subsequently reported in a series of in vitro and in vivo studies using VE-821 and VX-970 (M6620). IR alone induced HRR in a number of cancer cell lines, which was blocked by treatment with VE-821. This inhibition of HRR by VE-821 was associated with elevated DNA damage (measured by H2AX and 53BP1 foci) consistent with failed repair and the persistence of unrepaired damage. In clonogenic viability assays VE-821 significantly enhanced IR toxicity in a number of cancer cells, with changes in surviving fractions of about two to sixfold (for the combination vs. IR alone) (Prevo et al. 2012). This observation was confirmed in a second independent study that showed enhanced IR toxicity in a panel of 12 cancer cell lines, with substantial decreases in surviving fraction observed on treatment with VE-821 + IR vs. IR alone (Pires et al. 2012). Consistent with observations from combinations of ATR inhibitors and DNA damaging chemotherapy, it was shown using VX-970 (M6620) that non-cancer cells are able to tolerate the combination of an ATR inhibitor and IR with no enhanced toxicity (Fokas et al. 2012). Notably, it has also been shown that ATR inhibition can substantially radiosensitise hypoxic cancer cells (Pires et al. 2012). This is an interesting and potentially important observation since tumour hypoxia is a major barrier to successful responses to IR in patients (Pires et al. 2012).

Combinations of ATR inhibition and IR have been studied in a number of mouse xenograft models. Mice bearing either PSN1 or MiaPaCa-2 pancreatic tumours were treated with a single dose of IR ± 6 contiguous daily doses of VX-970 (M6620). In both models remarkable anti-tumour activity was observed for the combination, in contrast to either agent alone. Most impressive was the response in MiaPaCa-2 tumours, where sustained regression was observed in the combination treated group (Fokas et al. 2012). Marked anti-tumour activity was also observed for the combina-

tion in a third model when the IR was given using a fractionated regime: IR given as 5 daily doses of 2 Gy each, with VX-970 (M6620) given for 6 contiguous days starting 1 day prior to IR treatment. Anti-tumour activity was associated with a decrease in pCHK1 levels in tumours of IR treated mice, consistent with an ATR mediated mechanism of action (Fokas et al. 2012). In many clinical situations IR treatment is associated with concurrent chemotherapy, and the impact of ATR inhibition with such a treatment was assessed in a PSN-1 mouse xenograft. In this model the combination of VX-970 (M6620) with gemcitabine and IR was markedly more effective than any of the agents alone or gemcitabine plus IR. Notably, in all the models adding VX-970 (M6620) to either IR alone or IR and gemcitabine was well tolerated with no greater body weight loss when compared with control animals treated in the absence of VX-970 (M6620, Fokas et al. 2012). Importantly, the tolerance of normal tissues to VX-970 (M6620) and IR was assessed in tumour bearing mice irradiated ± VX-970 (M6620) treatment through the small bowel and the normal tissue assessed for evidence of intestinal cell death or adverse morphological changes. Treatment with IR alone led to increased TUNEL-positive apoptotic jejunal cells, that was not further increased by VX-970 (M6620). Furthermore, whereas IR alone induced both villus tip loss and villi shortening, neither was enhanced by the addition of VX-970 (M6620, Fokas et al. 2012). These data are consistent with in vitro findings that inhibition of ATR does not increase cell death in non-cancer cells exposed to DNA damaging agents such as IR.

The pre-clinical data demonstrating that ATR inhibition can markedly potentiate the anti-tumour activity of IR in a wide range of cancer models with minimal impact on normal tissue, and furthermore that ATR inhibition can sensitise hypoxic cancer cells to IR (a common mechanism for IR resistance) provides a compelling rationale to test ATR inhibitors with IR in the clinic. A number of clinical studies are ongoing to assess both AZD6738 and VX-970 (M6620) with IR alone or as part of a chemoradiation therapy (Reviewed in Chap. 5).

4.6 ATR Inhibition as Monotherapy

Tumour DNA is in a more fragile state than in normal cells, leading to elevated background RS, a hallmark of cancer (Macheret and Halazonetis 2015). This can arise for example from dysregulated proliferation and loss of checkpoint control, and elevated levels of oxidative damage (due to mitochondrial dysfunction, altered metabolism and inflammation (Wiseman and Halliwell 1996; Storz 2005; Babior 1999; Berasain et al. 2009)). Accordingly, given the established apical role ATR plays in regulating the cellular responses to RS, there is much interest in the potential for ATR inhibitors to be used as single agents. This could be exacerbated in cells that concurrently carry defects elsewhere in the DNA repair network, placing further reliance on ATR. Both these concepts are discussed below.

Endogenous events that drive RS: Many transforming oncogenes such as K-ras or C-myc act to drive dysregulated S-phase entry and their expression is

widespread across cancer. The resulting oncogenic stress has been shown to elevate RS and activate ATR. This can be attributed to a series of events including premature origin firing, exhaustion of the nucleotide pool, oxidative DNA damage and potential clashes between replication and transcription machinery (Davidson et al. 2006; Moiseeva et al. 2009; Dominguez-Sola et al. 2007). The potential for tumours driven by such oncogenes to be dependent on ATR for survival and thus be sensitive to ATR inhibition has been characterised in a number of studies. Transformation of mouse embryonic fibroblast (MEF) cells with either K-ras or H-ras led to marked elevation of ATR activity consistent with elevated RS. Hypomorphic suppression of ATR by shRNA (>80% depletion of ATR protein) led to potent suppression of cell growth and elevated cell death in the transformed cells (Gilad et al. 2010). Similarly, Myc transformation led to elevated RS in MEFs: DNA damage was further enhanced following ATR depletion, which was associated with increased cell death (Murga et al. 2011). Furthermore, shRNA for ATR significantly reduced the viability of Myc upregulated multiple myeloma cells, which was attributed to Myc-induced oncogenic stress and increased reactive oxygen species (ROS). Loss of cell viability was increased by ROS induction using piperlongumine (Cottini et al. 2015) and the sensitivity of Myc transformed cells to ATR depletion was enhanced in p53-deficient cells, consistent with a model in which blockade of compensatory DDR signaling or G1 checkpoint control augments reliance on ATR (Murga et al. 2011). In subsequent studies, oncogene transformation was shown to sensitise cells to inhibition of ATR. Using an analog of VE-821, inhibition of ATR in H-ras or C-myc transformed MEFs increased S-phase DNA damage (γH2AX), the frequency of chromatid breaks, cell growth inhibition and cell death, relative to the impact of the ATR inhibitor in non-oncogene transformed matched cells (Schoppy et al. 2012). Another cell cycle regulator that is commonly amplified in cancer is Cyclin E1: amplification is observed in some cancers such as high-grade serous ovarian cancer. Cyclin E forms a complex with cdk2 to promote S-phase entry (Patch et al. 2015) Inhibition of ATR by ETP-46464 led to substantial elevation of RS in Cyclin E transformed MEFs vs. untransformed cells. The synthetic addiction of Cyclin E1 amplification with ATR inhibition was markedly enhanced in the absence of p53 (Toledo et al. 2011).

In addition to the expression of oncogenes driving dysregulated proliferation and its associated RS, a number of other cancer relevant mechanisms have been shown to elevate RS. Perhaps the most intuitive are defects that impair the DNA replication machinery. The result would be a potential uncoupling of the helicase and the replicase complex, leading to exposed ssDNA. Several synthetic lethal screens using either selective ATR inhibitors or cells expressing the ATR Seckel mutation (associated with substantial reduction of ATR) have shown that silencing of some genes involved in DNA replication is synthetically lethal with ATR inhibition or depletion. Of note, silencing of the RRM1 and 2 genes that form the ribonuclease reductase enzyme (responsible for synthesizing the nucleotide DNA building blocks), PRIM1 that makes the RNA primers required for the lagging strand replication, and POLD1 the DNA polymerase responsible for lagging strand replication, all resulted in marked sensitivity to ATR depletion or inhibition (Hocke et al. 2016; Mohni et al. 2015). In the case of POLD1 more detailed studies showed reducing ATR activity

both through transfection of the ATR Seckel gene or inhibition with VX-970 (M6620) increased sensitivity of DLD1 cells to POLD1 silencing by nearly tenfold. In a second experiment, depletion of POLD1 by siRNA in a panel of cell lines increased the growth inhibitory activity of VX-970 (M6620) by up to tenfold. This was associated with increased RS and cell death (Hocke et al. 2016). Missense mutations in the POLD1 gene have recently been identified in colorectal, endometrial, brain and renal cancer (Hocke et al. 2016).

Regions of hypoxia are a hallmark of solid tumours, arising as a result of an inefficient tumour vasculature. Tumour hypoxia leads to a repression of DNA repair pathways and as a consequence an increase in genomic instability. Severe hypoxia also leads to elevated RS, which has been attributed to acute depletion of nucleotide pools, most likely through impairment of oxygen-dependent ribonucleotide reductase activity. Given the elevated RS that accompanies hypoxia it is perhaps unsurprising that ATR activity has been shown to be increased under these conditions (Pires et al. 2012). As such, hypoxia sets up an environment where RS is elevated but where many DNA repair pathways are repressed, a seemingly perfect scenario for dependence on ATR. This was first assessed using siRNA depletion of ATR: hypoxic cells depleted of ATR showed increased cell death compared with control treated cells (Hammond et al. 2004; Hammond and Giaccia 2004). Using VE-821 it was subsequently demonstrated that inhibition of ATR also sensitises cells to hypoxia. Treatment of hypoxic RKO cells with VE-821 led to a substantial decrease in P-CHK1 and a concomitant increase in DNA damage. This was associated with a marked decrease in clonogenic survival in VE-821 treated RKO cells exposed to short periods of hypoxia followed by reoxygenation. Sensitivity to the ATR inhibitor was both dependent on oxygen tension (increased hypoxia leading to greater sensitivity) and the duration cells were left under hypoxic conditions (Pires et al. 2012).

Finally, telomere maintenance is essential for cancer cells to attain immortality and whereas most cancer cells use the telomerase machinery to maintain telomeres, a sub-set of cancer cells use an HRR-dependent process known as Alternative Lengthening of Telomeres (ALT) (Draskovic and Londono-Vallejo 2014). Telomeres also represent hard to replicate, fragile, regions of the genome, which is partly associated with prevalent G-rich hexameric TTAGGG repeats. In ALT positive cells this situation is even worse since ALT telomeres comprise a series of variant hexameres, which disrupt the binding of important telomeric capping proteins. As a consequence, replication of ALT telomeres leads to high levels of RS (Cox et al. 2016). Using VE-821 it has been shown that inhibition of ATR leads to rapid loss of telomeres in ALT positive cancer cells and cell death after just one or two rounds of cell cycle. The IC_{50} values for inhibition of cell viability by VE-821 in ALT positive cell lines were on average over tenfold lower than a similar set of telomerase (ALT negative) cell lines. In addition to compromising the cell response to RS generated during the replication of ALT telomeres, ATR inhibition blocked the process of ALT itself (Flynn et al. 2015). Intriguingly however, in a second independent study, ALT positive cells were not found to be especially more sensitive to the ATR inhibitor VE-821 than cells utilizing a telomerase mechanism (Deeg et al. 2016).

Further studies are clearly required to determine the potential for ATR inhibitors as monotherapy in ALT tumors.

Endogenous events that impair DNA repair driving a reliance on ATR for survival: In addition to events that elevate RS, sensitivity to ATR inhibition as a monotherapy has been shown to be affected by defects elsewhere in the DNA repair network. Interestingly, this sensitivity can arise from defects in proteins associated with the ATR pathway along with defects in alternative surveillance and repair pathways.

In two synthetic lethal screens using VE-821, the strongest hits were with genes on the ATR pathway: ATR itself, ATRIP, RPA, Claspin, Hus1, Rad1 and CHK1 (Mohni et al. 2015, 2014). In subsequent studies, depletion of ATR or CHK1 increased sensitivity of U2OS cells to VE-821 by up to ~5-fold. Interestingly the presence of a heterozygote ATR mutant (on one of the two alleles) was sufficient to sensitise the cells to VE-821. These findings can be interpreted in a number of ways, either partial suppression of the ATR pathway places a greater reliance on the residual capacity and thus increases sensitivity to ATR inhibition; or it could be that the signaling pathway isn't always linear, for example, independent signals may lead to regulation of different ATR pathway proteins or the pathway may comprise regulatory feedback processes (discussed below for CHK1). Regardless of the underlying mechanism, the observation of synthetic lethality between ATR inhibition and depletion of genes on the ATR pathway highlights an interesting opportunity for use of ATR inhibitors as single agents since up to 25% of some cancer types harbor mutations or deletions in ATR pathway genes (Cerami et al. 2012).

RPA (replication protein A) is rapidly recruited to single stranded DNA where it protects it from nuclease cleavage and also recruits ATRIP and ATR. In an elegant in vitro study (Toledo et al. 2013) it was demonstrated that cells express a defined pool of nuclear RPA. When activated at a stressed fork, ATR signals to shut down global origin firing via CHK1, which acts to limit the number of stressed forks and thus depletion of the RPA pool. However, when ATR is inhibited DNA replication continues and RPA is depleted as the number of stalled forks increase. Once the RPA pool is exhausted the exposed, unprotected single stranded DNA at a stalled fork is rapidly converted to a double strand break. Consistent with this model, it was shown that RPA provides a resistance mechanism to ATR inhibition: overexpression of RPA by two to threefold was sufficient to protect cells from ATR inhibition at the time points assessed in the study. Conversely, depletion of the RPA pool markedly enhanced the sensitivity of cells to ATR inhibition (Toledo et al. 2013). This study highlights an attractive potential opportunity for single agent ATR inhibition in tumours with limited RPA pools. Such a situation could arise either from low baseline levels of RPA expression or from a combination of low RPA expression and elevated background RS. Further studies are required to assess and validate this approach and to define the appropriate markers that could support clinical investigation.

Finally, defects in HRR, the repair pathway ATR signals to, have also been shown to confer sensitivity to ATR inhibitors as single agents. In one study, either depletion of the HRR essential protein RAD51 (a recombinase involved in the homology search and strand pairing aspects of HRR) or its inhibition by the compound BO2, rendered cells highly sensitive to ATR inhibition by VE-821. For example, in HeLa

cells VE-821 treatment alone led to about 75% clonogenic survival, however following RAD51 depletion by siRNA, viability was reduced to <5% (Krajewska et al. 2015). In a second study, cells defective in the HRR genes BRCA2 (involved in the recruitment of RAD51 to single stranded DNA) or XRCC3 (which complexes with RAD51 to effect homology search and strand pairing) were markedly sensitized to VE-821 compared with parental cells. Specifically, parental Chinese hamster ovary cells tolerated VE-821 with 91% clonogenic survival, in contrast cells defective in XRCC3 showed only 16% survival; and Chinese hamster lung cells with defective BRCA2 were also markedly sensitised compared with parental cells (8% survival compared with 38% survival, respectively) (Middleton et al. 2015).

In addition to defects in ATR pathway genes, loss of function in other DNA repair pathways has been shown to confer sensitivity to ATR inhibitors as single agents. Given that both the ATR and ATM mediated pathways signal to HRR in response to damage during S/G2 phases of cell cycle, and that loss of ATM signaling pathway function appears to sensitise cells to ATR in combination with DNA damaging agents (Cui et al. 2014), it is perhaps unsurprising that a number of studies have provided data that shows defects in ATM pathway signaling can confer sensitivity to single agent ATR inhibition. This was first demonstrated by Wright et al. (Wright et al. 1998) based on the observation that the ATR-kinase dead mutation led to markedly reduced viability of cells with mutant p53 or ATM deficiency. More recently, siRNA of ATM in U2OS cells led to increased sensitivity to the ATR inhibitor AZ20 by almost five-fold (Lee et al. 2011). ATM loss due to deletion of the 11q22-23 locus or promoter methylation has been described in a number of diseases including head and neck squamous cell carcinoma (HNSCC), mantle cell lymphoma (MCL) and chronic lymphocytic leukemia CLL (Lee et al. 2011; Menezes et al. 2015; Boultwood 2001; Schaffner et al. 1999). Treatment of two MCL lines, one with and one without deletion of the 11q22-23 locus, showed a differential sensitivity to AZ20: the ATM wild type line tolerated high concentrations of AZ20 (<20% growth inhibition at 1 μM) in contrast to the ATM null line where substantial growth inhibition was observed (>90% at 1 μM). In a CLL study, ATM defective CLL cells (ATM shRNA depletion) were five-fold more sensitive to treatment with AZD6738 than the ATM wild type parental cells, and similarly, in a panel of 29 primary CLL samples ATM defective (n = 8) or *TP53* defective (n = 6) samples were more sensitive to AZD6738 than ATM/*TP53* wild type cells (EC_{50} 8.7 μM and 8.2 μM vs. 38.3 μM respectively) (Kwok et al. 2016). In mouse patient derived CLL xenograft studies using samples defective in either ATM or *TP53*, treatment with AZD6738 led to marked reduction in the number of CLL cells in the spleen of the mice. In one experiment tumour cell recovery was observed following treatment with AZD6738 and it was noted that the spleens of these mice had a significantly reduced frequency of ATM deficiency compared with the vehicle treated mice, supporting the hypothesis that ATR inhibition can be an effective approach to kill ATM or *TP53* defective tumour cells (Kwok et al. 2016). Interestingly, of the three reported mouse xenograft models based on solid cancer cell lines, where single agent ATR inhibition (AZ20 or AZD6738) has been shown to be effective, two are defective in ATM (Granta519 and LoVo) (Foote et al. 2015; Menezes et al. 2015).

BER and NER act to resolve small and bulky lesions respectively on nucleotides, and defects in BER and NER are widely reported in a variety of cancer types (Wallace et al. 2012; Marteijn et al. 2014). The X-ray repair cross-complimentary gene 1 (XRCC1) protein is a scaffold protein that plays an important role in recruiting key proteins for both BER and NER. (Caldecott 2003; Moser et al. 2007) In three separate studies it has been shown that isogenic cell pairs deficient in XRCC1 are more sensitive to ATR inhibition than their parental counterparts. The semi-selective ATR inhibitor NU6027 reduced clonogenic survival of XRCC1 null CHO derivatives by over 50% in contrast to parental cells, which tolerated the compound well (Peasland et al. 2011). This was associated with a marked increase in apoptosis (Sultana et al. 2013). Similarly, treatment of XRCC1 defective CHO cells with VE-821 led to marked inhibition of clonogenic viability (75%) in stark contrast to parental cells that tolerated VE-821 with minimal impact on survival (<10% loss of viability) (Middleton et al. 2015). Furthermore, inhibition of PARP, a key enzyme in BER, has been shown to dramatically sensitize cells to ATR inhibition (described in detail below).

The ERCC1-XPF nuclease complex functions in a number of repair pathways that act to resolve bulky DNA adducts, double strand breaks and interstrand cross links. Low levels of ERCC1 have been described in some cancers, most notably testicular cancer (Usanova et al. 2010). Depletion of ERCC1 by siRNA in five cell lines increased cell sensitivity to VE-821 in all cases, with IC_{50} shifts for VE-821 of up to 1 order of magnitude. This was associated with elevated DNA damage (by γH2AX) (Mohni et al. 2014). Finally, disruption of proteins in involved in NHEJ was shown to sensitise cells to ATR inhibition. Depletion of Ku80, a protein that binds DSBs and recruits DNA-PK to form the catalytically active enzyme required for NHEJ, sensitised CHO cells to VE-821 with just 20% clonogenic survival compared with >90% for the parental CHO cells. Intriguingly however, loss of DNA-PKcs itself rendered CHO cells marginally *resistant* to VE-821, and overexpression of DNA-PKcs in both human GBM and CHO cells that lacked DNA-PKcs increased their sensitivity to VE-821. Even more intriguingly, the effects of DNA-PKcs expression was not associated with catalytic activity since addition of the DNA-PK inhibitor NU7441 did not rescue the cells. The model proposed to rationalize these data was that elevated levels of DNA-PKcs led to increased loading and persistence of DNA-PKcs on DSBs. Since end resection, revealing regions of ssDNA and recruitment of ATR, is a key step in DSB resolution, persistence of DNA-PKcs may impair this process. The impact of this could be to reduce ATR signaling capacity, placing increased reliance on residual proficient ATR signaling and rendering cells highly sensitive to ATR inhibition (Middleton et al. 2015).

Taken together, the emerging picture is that somatic defects leading to elevated RS, and/or reliance on ATR through impairments in DNA repair processes, have the potential to render cells sensitive to ATR inhibition as a monotherapy. Whilst this provides an exciting opportunity, defining translationally robust markers that support monotherapy activity in the context of the heterogeneity of human cancer remains a very important task.

4.7 ATR Inhibition in Combination with Targeted Drugs

As discussed above, a number of genetic studies have demonstrated that depletion of genes involved in DNA repair can drive a reliance on ATR to survive DNA damage. Accordingly, there is potential for ATR inhibitors to provide benefit when used in combination with agents that block other proteins involved in the surveillance and response to DNA damage. Two examples have been described; ATR inhibition with PARP inhibition and ATR inhibition with CHK1 inhibition.

PARP is a key enzyme involved in the repair of single stranded DNA (ssDNA) breaks, primarily during BER. PARP is recruited to sites of ssDNA damage where it acts to add ADP-ribose moieties to proteins, a process termed PAR-ylation. The result is an increasingly negatively charged region at sites of damage that serves to recruit the BER multi-protein complex including proteins such as DNA ligase III, DNA polymerase beta and the scaffold protein XRCC1 (Curtin 2014). PARP has also been shown to play a role in regulating replication fork dynamics at sites of RS and a direct interaction between PARP and ATR has been demonstrated in response to DNA damage (Bryant et al. 2009; Sugimura et al. 2008; Kedar et al. 2008). PARP inhibition is synthetically lethal with loss of the HRR essential genes BRCA1/2 (Li and Yu 2015). Given these observations it was intriguing to consider whether ATR inhibition could sensitise cells to PARP inhibition. The combination of an ATR and PARP inhibitor was first described in 2011 with NU6027 and the PARP inhibitor rucaparib (Peasland et al. 2011). Rucaparib alone led to elevated DNA damage (γH2AX foci formation) and increased HRR pathway activity (RAD51 foci) in a BRCA wild type cell line. Co-treatment with NU6027 completely blocked RAD51 foci formation consistent with inhibition of HRR. In two different cell lines, both with functional HRR, NU6027 increased the cytotoxic activity of rucaparib: in GM847KD cells, expression of ATR kinase dead or treatment with NU6027 reduced the LC_{50} for rucaparab from >30 μM to about 12 μM, and in MCF7 cells clonogenic survival was reduced from 60% to 70% for rucaparib alone to about 20% on co-treatment with NU6027 (Peasland et al. 2011). ATR depletion and VE-821 also sensitized ovarian cancer cells to the PARP inhibitor, veliparib (Huntoon et al. 2013). In a subsequent study, reported at the AACR annual meeting in 2016, ATR inhibition using VX-970 (M6620) was shown to synergise with all the available clinical PARP inhibitors (veliparib, olaparib, rucaparib, niraparib and talazoparib) across a panel of cancer cell lines. Importantly, synergy was not observed in a non-cancer cell line. Furthermore, in isogeneic cell pairs, loss of either ATM or p53 resulted in marked sensitivity to the combination of VX-970 (M6620) and talazoparib. Consistent with this, across a large panel of over 100 cancer cell lines, greater synergy was observed for the combination of VX-970 (M6620) and talazoparib in cell lines with a mutation of the *TP53* gene (Pollard et al. 2016a, b). A similar profile was reported for AZD6738 and olaparaib, at the EORTC/NCI/AACR triple meeting in 2015 (Lau et al. 2015). These data are comparable with the observations for combinations of ATR inhibitors with cytotoxic chemotherapy and suggest that loss of the compensatory ATM-p53 signaling pathway may be a marker for tumour sensitivity.

Accordingly, activation of the ATM-p53 pathway may provide a mechanism to enable non-cancer cells to tolerate the combination. In vivo benefit for the combination has been reported with AZD6738 and olaparaib in two primary explant mouse xenograft models. In the first model, which was BRCA2 and *TP53* mutant, olparaib alone was active (partial tumour growth inhibition) consistent with the established synthetic lethality of PARP inhibition and BRCA mutation, whereas AZD6738 had no single agent activity. Impressively, the combination led to complete and sustained regression. In the second model, which was ATM and *TP53* mutant but BRCA wild type, the combination led to complete tumour growth inhibition in contrast to either agent alone, which were inactive (Lau et al. 2015). A number of clinical studies are actively assessing the combination of PARP and ATR inhibitors either as doublets or with the addition of chemotherapy (Chap. 5).

CHK1 and ATR function in the same pathway to coordinate cell responses to DNA damage. However, there are circumstances where each appears to function independently. For example, ATR has been reported to control the intra S-phase checkpoint independently of CHK1 activity (Couch et al. 2013; Luciani et al. 2004), and conversely CHK1 has been reported to be activated in response to RS by claspin in an ATR independent manner (Yang et al. 2008). Furthermore, differences in the potential for CHK1 and ATR inhibition to sensitise cells to various DNA damaging drugs was demonstrated from a large panel of lung cancer cell lines. Whereas inhibition of either CHK1 or ATR sensitised many cancer cells to gemcitabine, inhibition of CHK1 had only a moderate impact on cancer cell sensitivity to platinating agents in contrast to ATR inhibition, which induced substantial cell sensitivity (Hall et al. 2014). The potential for ATR and CHK1 inhibitors to provide a beneficial combination therapy was characterized in a series of elegant studies. Against a panel of seven cancer cell lines co-treatment with the CHK1 inhibitor AZD7762 and the ATR inhibitor VE-821 led to synergistic loss of viability in all seven lines. A subsequent experiment in a sub-set of the cell lines showed that AZD7762 reduced the IC_{50} of VE-821 by three to tenfold (Sanjiv et al. 2016). In contrast, the two agents when combined did not impact the viability of a number of non-cancer cell lines. To address a concern that the combination is merely a hypermorphic response i.e., that the combined effect arises through more comprehensive inhibition of the pathway than can be achieved by either agent alone, CHK1 null DLD1 cells were treated with VE-821. Almost complete loss of clonogenic survival was observed (<5% survival), in stark contrast to parental cells that were tolerant to very high concentrations of VE-821 (>70% clonogenic survival). The cytotoxic activity of the combination appeared to be dependent on RS since Myc expressing cells were acutely sensitive to VE-821 plus AZD7762, as opposed to parental non-transformed cells that were resistant to the combined drug treatment. Detailed molecular studies led to the proposal of a model in which CHK1 inhibition leads to a CDK mediated increase in origin firing, which in turn leads to depletion of the dNTP pool, slowed or stalled fork progression, increased levels of RS and a concomitant reliance on ATR. Interestingly, this model suggests that CHK1 could limit the efficacy of ATR inhibition and vice versa. The potential benefit for combined treatment with ATR and CHK1 inhibition was assessed in a mouse H460 cell line xenograft. Treatment with either AZD7762 or VX-970 (M6620) alone had minimal impact on tumour cell

growth and survival, however the combination resulted in almost complete tumour growth inhibition and a marked increase in survival. The combination was well tolerated with no body weight loss. This study highlights an interesting intrapathway synthetic lethality that could be exploited to provide a tumour specific anti-cancer therapy (Sanjiv et al. 2016).

4.8 Conclusion

The ATR kinase plays an important role in the cells response to exposed ssDNA, a structure most commonly formed at stalled replication forks (replication stress, RS), but also as an intermediate in a number of repair processes. In the absence of a functional ATR response, unresolved ssDNA can form a lethal DSB. RS and ssDNA can result from many types of DNA damage insult including endogenous events such as oxidative stress and deregulated DNA replication (for example from expression of oncogenes); or from exogenous events such as hypoxia or treatment with DNA damaging chemotherapy or IR. Elevated levels of ssDNA drive an acute reliance on ATR, which can be further exacerbated in cells where alternative DNA damage repair processes are impaired. Both high levels of DNA damage and defective DNA repair are hallmarks of cancer and numerous genetic and pharmacologic studies have demonstrated that many cancer cells are reliant on ATR to survive DNA damage. Inhibition of ATR is frequently lethal to cancer cells either alone or when treated in combination with agents that induce DNA damage. In contrast non-cancer cells can tolerate ATR inhibition through activation of a compensatory DNA damage response. Given the multiple contexts in which RS can be elevated in cancer cells, there are many opportunities where ATR inhibitors have the potential to provide patient benefit. Perhaps the best pre-clinically validated opportunity is as a combination therapy with DNA damaging drugs and IR. This, coupled with the widespread role these agents play in standard of care across multiple cancer types and the emerging role DNA repair has as a clinically relevant mechanism of resistance to such agents, has led to growing interest in the numerous ongoing clinical studies assessing ATR inhibitors with various DNA damaging drugs and IR. A growing body of evidence also supports a potential for ATR inhibitors as monotherapy in cancers with high levels of background DNA damage and/or defects in compensatory repair pathways; and as combination therapies with agents that block other DNA repair processes.

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