ATR as a Therapeutic Target

Fiona K. Middleton and Nicola J. Curtin

1 An Introduction to ATR

Ataxia Telangiectasia Mutated and Rad3-related (ATR) is a vital sensor of a variety of DNA lesions and is critical to cell cycle arrest at the S and G2 checkpoints as well as initiation of DNA repair via homologous recombination repair (HRR). 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) [1]; protein kinases that are also involved in the complex network of DNA damage signalling and repair mechanisms known as the DNA damage response (DDR). The DDR comprises sensor proteins which detect the DNA damage and signal to transducer proteins, e.g. p53 and checkpoint kinases which then transmit this information to downstream effector proteins. These effectors activate the appropriate damage response, be it cell cycle arrest and DNA repair or apoptosis. Many of the phosphorylation substrates of ATR are also common to ATM, and the two are both involved in HRR in response to double strand breaks (DSBs). There is also crosstalk between the two PIKKs. ATM and ATR phosphorylate >900 sites on >700 proteins in response to DNA damage induced experimentally, highlighting the complexity of the network. The majority of phosphorylated proteins are involved in DNA replication, recombination and repair plus cell cycle regulation [2].

F.K. Middleton • N.J. Curtin (✉)
Northern Institute for Cancer Research, Newcastle University,
Paul O'Gorman Building, Framlington Place, Newcastle upon Tyne NE2 4HH, UK
e-mail: nicola.curtin@newcastle.ac.uk

L. Panasci et al. (eds.), *Advances in DNA Repair in Cancer Therapy*,
Cancer Drug Discovery and Development 72, DOI 10.1007/978-1-4614-4741-2_10,

1.1 Investigating the Role of ATR

The first indications that ATR may be involved in cell cycle checkpoints came from the cloning of ATR using homology sequencing with Rad3 of *S. pombe* , which is known to be involved in cell cycle arrest [3]. The critical role of ATR for viability was established when it was discovered that $ATR^{-/-}$ mice die on embryonic day 7 [4]. This may explain why no humans with germ-line homozygous ATR deletions have been identified, which, for a long time, made characterising ATR challenging. Blastocysts from the $ATR^{-/-}$ mice could be harvested prior to death, cultured and analysed. This showed that this embryonic lethality was due to increased apoptosis and chromosomal fragmentation [4]. Such fragmentation is also observed in cells undergoing mitotic catastrophe; a characteristic of cells entering mitosis prematurely following incomplete DNA synthesis [5]. This was one of the first indications that ATR is critical to cell cycle arrest.

In humans, the only well characterised disorder associated with ATR is the autosomal-recessive disease Seckel syndrome [6]. Sufferers have a hypomorphic mutation in the ATR gene resulting in low levels of the protein and exhibit growth retardation and microcephaly; characteristics which are similar to those exhibited by sufferers of other disorders associated with impaired cell cycle arrest such as Nijmegen breakage syndrome [7]. Examination of lymphoblast cell lines from Seckel patients has shown that they exhibit a three- to seven-fold increase in chromosomal fragmentation following inhibition of DNA synthesis [8] which is similar to that seen in $ATR^{-/-}$ murine cells [4]. Interestingly, Seckel syndrome patients do not have an increased prevalence of cancer, which is surprising as in vivo studies showed $ATR^{+/-}$ mice have an increased incidence of tumour formation [4].

The role of ATR in cell cycle arrest was confirmed upon the development of cells expressing an inducible ATR kinase-dead (ATR-KD) cell line where the ATR-KD acts as a dominant negative inhibitor of the native protein [9]. When ATR was no longer active, these cells did not arrest following DNA damage and, in particular, lacked G2 arrest [9]. Further work in ATR-KD cells showed that ATR becomes active following different types of DNA damage compared to ATM [10, 11].

1.2 ATR: A Sensor of DNA Damage

ATR is activated by the single stranded–double stranded DNA (ssDNA–dsDNA) junctions, which arise principally at stalled replication forks, resected double strand breaks (DSBs) and nucleotide excision repair (NER) intermediates. Stalled replication forks occur when the dNTP pool is depleted preventing further DNA synthesis, when the number of origins of replication exceeds dNTP supply or when the replication machinery encounters a DNA lesion. This tends to occur when the advancing replication fork reaches lesions such as single-strand breaks (SSBs), bulky adducts and interstrand cross links (ICLs) [12, 13]. Under these

circumstances the polymerase on the damaged strand may arrest while the opposing polymerase continues [14], thus creating the ssDNA–dsDNA structure. Many of these lesions occur endogenously; reactive oxygen species (ROS) are the major source of the 10^4–10^5 base lesions that are generated per cell each day [15] which can lead to SSBs and also the 50 DSB generated/cell/day [16]. Bulky adducts and ICLs can be created by environmental mutagens such as UV light and tobacco smoke [17].

The nucleotide excision repair (NER) pathway is used to remove bulky adducts such as UV-induced (6–4) photoproducts [11], which occur on a single strand of the DNA [18]. These adducts are removed by cleaving away 20–30 nucleotides of DNA around the damage, leaving a portion of ssDNA and thus generating the ssDNA–dsDNA structure.

Resected double strand breaks (DSBs) also create the ssDNA–dsDNA structures that activate ATR [19]. A major cause of DSBs is ionising radiation (IR). IR has been shown to primarily activate ATM rather than ATR. ATM is recruited to DSBs by the MRN complex (composed of Mre11, Rad50 and Nbs1) and upon its activation stimulates resection by phosphorylating the MRN complex and EXO [20]. The exonuclease activity then acts on one of the DNA strands causing the ssDNA–dsDNA structure to form, thus activating ATR [20]. In vitro work has shown that TopBP1, an important component in HRR, interacts with Nbs1 [21] and TopBP1 feeds back to activate ATR [22].

ATR activation is initiated by the binding of the single-stranded binding replication protein A (RPA) to the single-stranded portion of the ssDNA–dsDNA junction [23]. The role of RPA is twofold: to protect the single-stranded DNA overhang from exonuclease activity thus preventing formation of lethal DSBs, and to activate the downstream ATR pathway to initiate cell cycle arrest and DNA repair. RPA interacts with the ATR interacting protein ATRIP which is in complex with ATR, thus recruiting ATR to the DNA [24, 25]. The Rad17-RFC complex is simultaneously recruited to the DNA [26] mediating loading of the Rad9-Rad1-Hus1 (9-1-1) complex onto the DNA and thus recruiting TopBP1. TopBP1 is ultimately responsible for the activation of ATR kinase [22]. Active ATR kinase can then signal to downstream transducer proteins.

The mismatch repair system (MMR) is also important in ATR activation. MMR repairs any DNA base mismatches or insertion deletion loops that most often occur due to alkylating mutagens [18]. O^6-methylguanine is a common result of exposure to alkylating agents and is a major target of the MMR machinery. Key protein complexes within this repair system are the MutSα and MutLα complexes which are required for recognition and excision of mis-incorporated bases (including O^6-methylguanine). Evidence has shown that the ATR-ATRIP complex is recruited to O^6-methylguanine in a MutLα and MutSα-dependent manner and that this recruitment activates ATR [27]. The MSH2/MSH6 heterodimer binds the mismatched DNA, and immunoprecipitation experiments showed that ATR interacts with both of these component proteins. Furthermore, siRNA depletion of MSH2 has demonstrated that MSH2 is required for CHK1^{ser317} phosphorylation [28]. There is additional evidence of the interaction between the MMR machinery and

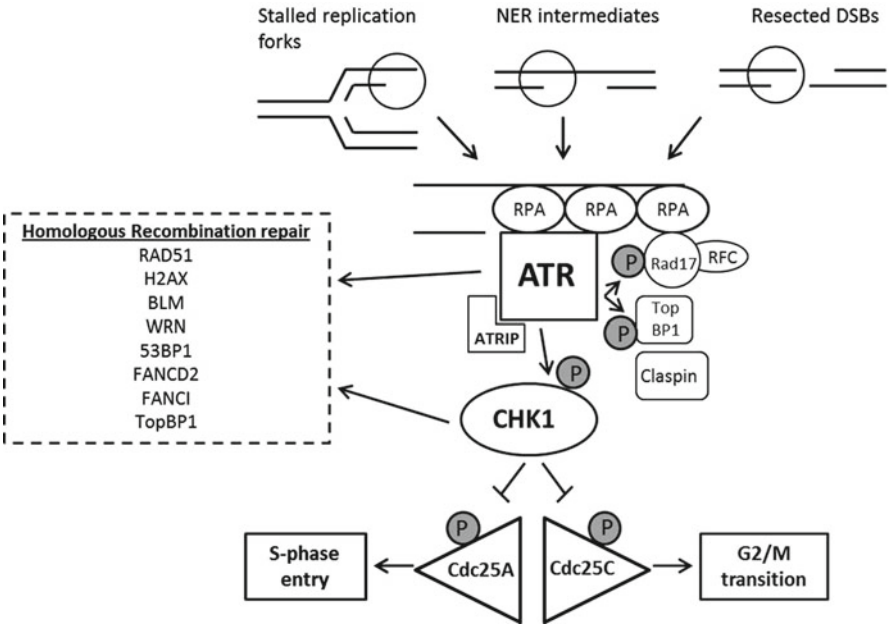

Fig. 1 Role of ATR in S/G2 checkpoint control and DNA DSB repair via homologous recombination. ATR-ATRIP is recruited by RPA to ssDNA–dsDNA junctions. The Rad17-RFC complex is also recruited to the DNA leading to TopBP1 recruitment and ultimate activation of ATR kinase. ATR phosphorylates a number of downstream targets, the best described of which is CHK1. CHK1 becomes active and phosphorylates downstream targets including the Cdc25 phosphatases. Cdc25A mediates S-phase entry by dephosphorylating CDK2/Cyclin A and Cdc25C controls G2/M transition via dephosphorylation of Cdc2/Cyclin B. Both ATR and CHK1 interact with and phosphorylate proteins involved in HRR repair

the ATR pathway as MutSα interacts with ATR, CHK1 and TopBP1 in a purified system [29]. MMR-proficient cells also form ATR foci following DNA damage with the alkylating agent temozolomide (TMZ) whereas their MMR-deficient counterparts do not [30].

1.3 Involvement of ATR in S/G2 Arrest

To prevent any damaged DNA being replicated, the cell must first arrest its cell cycle in order to repair this damage. ATR kinase is pivotal to arrest at the S and G2 checkpoints (Fig. 1). A key downstream target of ATR is CHK1. When Rad17 is recruited to the DNA, it interacts with claspin [31]; a protein responsible for the regulation of CHK1 phosphorylation by ATR. CHK1 is an essential kinase [32] that is phosphorylated by ATR on serine residues 317 and 345. Phosphorylation of CHK1^{ser345} by ATR is essential for CHK1 kinase activation [33] and this phosphorylation event is often

used as a marker of ATR activity [34–36]. Upon phosphorylation at these residues CHK1 becomes active triggering autophosphorylation at serine 296 [36]. CHK1 then dissociates from the chromatin [37] and can phosphorylate a number of targets. Cell cycle arrest is achieved by phosphorylation of the cell cycle Cdc25 checkpoint phosphatase proteins rendering them inactive [38]. Inactive Cdc25A dephosphorylates the CDK2/Cyclin A or E complex that promotes S-phase entry [39]. Similarly, Cdc25C activates the Cdc2/Cyclin B complex by removing the inactivating phosphates on threonine 14 and tyrosine 15 of Cdc2 [39] to promote entry into mitosis. These two residues are phosphorylated by Wee1 [40]. The effect of CHK1 on cell cycle progression is therefore threefold: Cdc25A is phosphorylated and inactivated preventing S phase arrest, Wee1 is phosphorylated and stabilised [40] resulting in phosphorylation of Cdc2 at Thr14 and Tyr15, and Cdc25C is phosphorylated and inactivated allowing Cdc2 to remain phosphorylated thus preventing the G2/M transition.

1.4 ATR's Response to Endogenous Damage

While much of the research surrounding ATR concentrates on its role following exogenous DNA damage, it is important to remember that much of the damage incurred by the cell is from endogenous or environmental sources. As previously mentioned, ROS generated from metabolism are responsible for a plethora of lesions that occur on the DNA [15]. Skin cells are also exposed to UV radiation on a daily basis creating bulky adducts [11]. Evidence has shown that ATR is also vital in the absence of exogenous genotoxic stress [41]. This was initially demonstrated in cells from the blastocysts of $ATR^{-/-}$ mice where 60–65% of mitotic spreads contained fragmented chromosomes compared to the 0–2% seen in $ATR^{+/+}$ and $ATR^{+/-}$ mice [4]. Investigations have shown that ATR is associated with chromatin in normal proliferating cells to a greater extent at the S phase of the cell cycle [23] where the chromatin is most vulnerable to fragmentation. ATR has been shown to maintain fragile site stability as siRNA knockdown of ATR or expression of an ATR-KD mutant increases the average number of chromosomal breaks per cell when DNA replication is reduced using the replication inhibitor aphidicolin [42]. Furthermore, many of the proteins associated with ATR and its activation such as Rad17, TopBP1 and claspin are also associated with chromatin during unperturbed S-phase [43].

1.5 ATR and DNA Damage Repair

As well as arresting the cell cycle at the S and G2 checkpoints, ATR is pivotal to stabilising replication forks and initiating DNA damage repair (Fig. 1). This repair is primarily via HRR; however, there is considerable crosstalk between ATR and other

DNA damage repair pathways [41]. Some of this crosstalk is due to the formation of the ssDNA–dsDNA junction in repair pathway intermediates, as previously mentioned in the case of NER. ATR interacts with machinery from other pathways such as the non-homologous end joining (NHEJ) pathway, where ATR phosphorylates DNA-PK_{CS} [44], and the ICL and translesion synthesis (TLS) pathways in which ATR is activated by RPA and phosphorylates FANCD2 allowing cross links to ultimately be removed [41, 45]. Removal of these cross links leaves behind a gap which must be repaired via HRR. Evidence has also shown that G2 arrest to correct the mismatch repair (MMR) substrate 6-thioguanine is ATR dependent [46], and that ATR may indirectly influence the base excision repair (BER) pathway [47].

The repair pathway in which ATR is most strongly associated is the HRR pathway which is also activated by ATM [48]. The precise role of ATR in HRR is yet to be confirmed; however, the plethora of experimental evidence surrounding ATR and other proteins implicated in HRR has demonstrated that it has a significant role in this DNA repair pathway. HRR is only active during S and G2-phases of the cell cycle as it relies on the use of the homologous sister chromatid as a template for DNA repair. It is a complex repair pathway in which the ssDNA overhang, generated at stalled replication forks or following the ATM-dependent resection of DSBs [49, 50], is rapidly coated with RPA, preventing DNA degradation and recruiting the ATRIP-ATR complex. ATM and ATR both phosphorylate BRCA1 stimulating its E3 ubiquitin ligase activity that is needed for activation of the G2 checkpoint [51]. BRCA2, which also interacts with PALB2 and BRCA1 [52] delivers RAD51 to the break and aids its displacement of RPA to form the nucleoprotein filament that can invade the complementary duplex DNA [53–55]. The sequence from this chromatid is then replicated at the point of damage, making this repair pathway error-free [18].

Phosphorylation of HRR proteins by ATR is critical to HRR proficiency. HRR assays in ATR-KD cells have shown that cells lacking ATR kinase function have reduced levels of HRR [56]. CHK1 is also vital for HRR. An HRR model system where only colonies that have undergone HRR survive was used to show that inhibition of CHK1 and ATR by UCN-01 and caffeine, respectively, reduces HRR by three- to fourfold. Inhibition of CHK1 by UCN-01 or siRNA knockdown of CHK1 also reduces RAD51 focus formation, further confirming the requirement of active CHK1 in HRR [57].

Histone H2AX is a substrate common to both ATR and ATM, and is required for DSB repair. Phosphorylation of H2AX (γ-H2AX) is a marker of DSB and occurs in an ATR-dependent manner following treatment with the replication-arresting agent hydroxyurea (HU) and also UV [58]. Investigations have indicated that H2AX facilitates HRR, as γ-H2AX foci co-localise with foci of repair proteins including RAD51, BRCA1 and 53BP1 [58, 59].

A number of other HRR-associated proteins are directly phosphorylated by ATR. These include BLM which is a RecQ helicase that is deficient in Bloom's syndrome; a condition that predisposes to cancer [60]. BLM has been shown to be directly phosphorylated by ATR, and co-localises with ATR, RAD51 and γ-H2AX in response to HU [61].

Another RecQ helicase—WRN, deficient in Werner's syndrome—also interacts with and is phosphorylated by ATR. WRN co-localises with the HRR proteins RAD51 and RAD54 in response to the DNA cross-linking agent Mitomycin C (MMC), and immunoprecipitates with ATR demonstrating a direct interaction between these proteins [62]. ATR, BLM, WRN and 53BP1 interact with one another to promote RAD51 foci formation [63, 64]. These interactions are dependent on ATR activity; siRNA knockdown of ATR prevents phosphorylation of BLM and reduces formation of 53BP1 foci in response to HU. BLM and 53BP1 no longer co-localise in cells with siRNA knockdown of CHK1 following the same treatment [63].

More recently, the role of ATR in phosphorylating proteins in the Fanconi anaemia pathway has been highlighted. These proteins are involved in removing DNA ICLs and initiating repair of the DNA via HRR [65–67]. Following treatment with MMC, cells with active ATR and ATRIP phosphorylate FANCI and FANCD2, the nuclease responsible for DNA incisions on either side of the cross link, and target it for ubiquitylation and degradation [45].

1.6 Importance of the Target in Cancer Therapy

ATR has long been thought of as a suitable target for anticancer therapy because of the variety of DNA lesions that activate it. Many of the anticancer agents that are in routine clinical use act by damaging the DNA either by causing bulky adducts that are repaired by NER, e.g. cisplatin, or by inducing DNA DSBs, e.g. IR or topoisomerase II poisons (such as doxorubicin, mitoxantrone or etoposide), or stalled replication forks. Stalled replication forks occur when nucleoside triphosphates (dNTPs) are in limited supply e.g., due to HU therapy or when unrepaired DNA lesions encounter the advancing replication fork. Such lesions may be (1) single base damage induced by DNA methylating agents such as temozolomide (TMZ) or dacarbazine (DTIC) or oxidative damage resulting from IR-induced ROS, (2) single-strand breaks induced by topoisomerase I poisons such as irinotecan or topotecan, (3) or the most difficult of all lesions to repair, ICLs, induced by cisplatin and the bifunctional alkylating agents. Since all these lesions trigger ATR to promote survival, inhibition of ATR should promote cell killing.

It is important that the enhanced cell killing is directed at tumour cells and not normal tissues otherwise there would be no patient benefit. There are two key characteristics of cancer that indicate that ATR is likely to be a highly attractive target for *selective* cancer therapy: (1) continuous proliferation and (2) dysregulation of their G1 control [68], making them reliant on their remaining S and G2 checkpoints (Fig. 2). This situation can be brought about by multiple mechanisms, for example, activation of oncogenes that drive proliferation or up-regulation of the cyclins and CDKs that promote S-phase entry and/or loss of tumour-suppressor genes such as p53 and Rb that control entry into S-phase. This means that cancerous cells are much more likely than normal cells to enter S-phase with

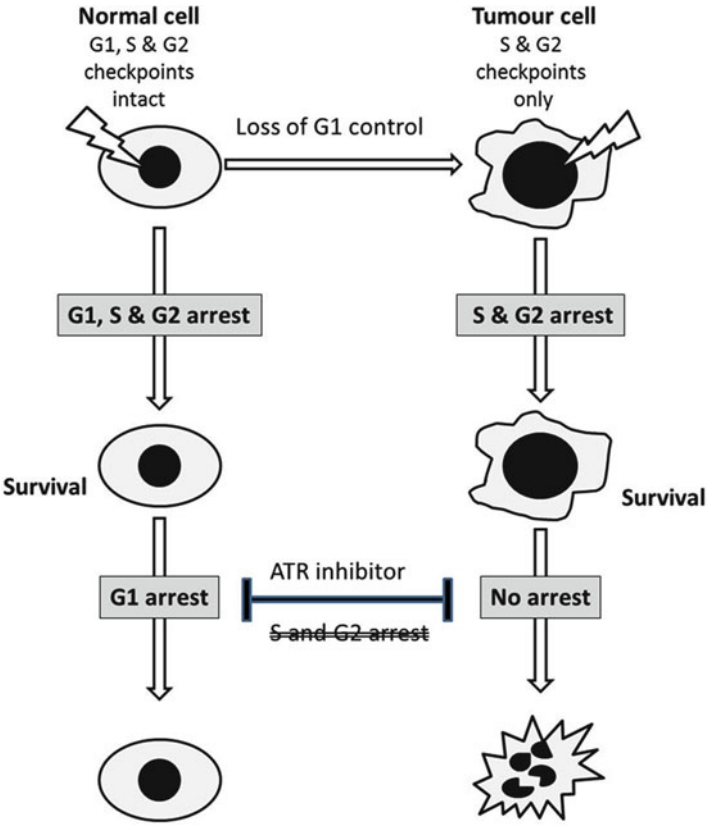

Fig. 2 Selective sensitization of cancer cells with dysfunctional G1 control by ATR inhibition. Loss of G1 control, e.g. by mutation of p53, frequently occurs during neoplastic transformation. While the normal cell (*left*) has all cell cycle checkpoints intact, the cancer cell relies on the S and G2 checkpoints it has retained. Both normal and cancer cells may be able to survive DNA damage (*lightening flash symbol*) by activation of cell cycle checkpoints. However, if ATR is inhibited the cancer cell will be unable to arrest and will die, but the normal cell may engage the G1 checkpoint and survive

damaged DNA or when appropriate biomolecules (e.g. dNTPs) are limited. Therefore, they have a high level of replicative stress, stalled replication forks and replication-associated DSBs. In addition, there are higher levels of ROS in tumours due to a number of factors, including increased metabolic activity, mitochondrial dysfunction, various oxidases [69, 70] and inflammation [71]. These give rise to approximately 100-fold higher levels of oxidative DNA lesions in tumours than in normal tissues [72]. It is apparent therefore that the DNA in a tumour cell is in a more fragile state. In response to this damage tumour cells lacking G1 control are much more likely to be dependent than normal cells on the S and G2 checkpoint function that they retain.

1.7 Validation of the Target by Genetic Inactivation

Abrogation of the S and G2 checkpoint has been the major rationale for the development of inhibitors of the ATR/CHK1 pathway. Whereas CHK1 inhibitors are in advanced clinical evaluation [73] ATR inhibitor development has lagged behind, which may reflect the difficulty of assaying an enzyme that requires a complex of co-activators and regulators [74]. Until recently, most of the studies demonstrating chemo- and radiosensitisation by ATR inactivation have used genetic manipulation. Two early independent "proof of principle" genetic studies with cells expressing ATR-KD mutants demonstrated abrogation of DNA damage-induced G2 arrest and sensitization of cells to a variety of DNA damaging chemotherapeutic agents [9, 75]. In the first study human SV40 transformed fibroblasts transfected with a doxycycline-inducible ATR-KD mutant were used. When the ATR-KD was expressed clonogenic survival following exposure to the DNA cross-linking agents cisplatin and MMC and the antimetabolite HU was profoundly reduced with a modest increase in UV sensitivity [9]. Further studies by this group also demonstrated sensitization to topoisomerase I poisons by activation of the ATR-KD in these cells [10]. In the second study, premature chromatin condensation (PCC) was used to demonstrate cell death in ATR-KD U2OS cells exposed to UV irradiation or HU [75]. These studies demonstrated that the previously observed enhancement of HU cytotoxicity by caffeine was due to ATR inhibition. Another approach used cells transfected with the Seckel mutant ATR that have very low levels of ATR activity. These cells were sixfold more sensitive to IR and the topoisomerase II poison doxorubicin, 10 to 20-fold more sensitive to the antimetabolites 5-Fluorouracil, gemcitabine, HU and methotrexate and >400-fold more sensitive to cisplatin than isogenic ATR expressing DLD1 cells [76]. ATR knockdown also caused a profound sensitization to cisplatin and gemcitabine in HeLa, HCT116 and U2OS cells [77]. ATR knockdown has also been shown to enhance the cytotoxicity of DNA methylating agents, such as TMZ, [78], and this may be dependent on a functional MMR pathway [30].

It has been postulated that targeting of the ATR/CHK1 pathway is only relevant in cells with defective G1 control through loss of the tumour suppressor gene p53. However, the selectivity of ATR inactivation may not be restricted to p53 defective cells. ATR silencing sensitised both HeLa (p53 defective) and U2OS (p53 wild-type) to topoisomerase I poisons [79]. It should be noted that U2OS cells do have an element of G1 dysfunction by virtue of p16 deletion, and following further impairment of the Rb pathway by over-expression of cyclin D, cyclin E or CDK2, there was enhanced UV-induced PCC in the ATR-KD cells, but not ATR-wt U2OS cells [75]. Inactivation of the p53 pathway in these ATR-KD U2OS cells, by MDM2 or human papilloma virus E6 expression, also increased the level of PCC threefold. This confirmed previous studies indicating that abrogation of G2 arrest and radiosensitisation by caffeine is greater in p53 null cells than p53 wt cells [80]. Sensitization was specific to replicating cells and selective to cells defective in the G1 checkpoint, although there is not a clear relationship to p53 status.

1.8 Development of Inhibitors

Caffeine (Fig. 3) was the first small molecule inhibitor of G2 arrest [81] to be identified as an inhibitor of ATR [82]; however it was weak (IC_{50} = 1.1 mM) and was a more potent inhibitor of the other PI-3K family members ATM and mTOR, and weakly active against DNA-PK. Radiosensitisation by caffeine was attributed to both ATM and ATR inhibition but UV sensitization was dependent on ATR inhibition rather than ATM inhibition [82]. Enhancement of UV-induced PCC was also specific to ATR [75]. Despite its lack of potency, its easy availability has led to several studies investigating abrogation of S and G2 checkpoints and chemo and radiosensitisation by caffeine. Caffeine also enhanced DNA damage and the cytotoxicity of TMZ in combination with IR in a glioblastoma model [83]. Schisandrin B (Fig. 3), a natural product, was identified as an inhibitor of ATR with an IC_{50} of 7.25 μM, and abrogated the UV-induced S and G2/M checkpoint and increased UV cytotoxicity in human lung cancer cells [84]. In a screen of PI-3K inhibitors PI-103 and PI-124 (Fig. 3) were identified as being more potent than previously identified ATR inhibitors with IC_{50} values of 0.9 and 2 μM, respectively [85]; however, these inhibitors have not been taken forward as ATR inhibitors due to their pan PI-3K family activity.

Recently, progress has been made on two fronts; firstly, by development of a high throughput cell-based screen to measure ATR activity and its inhibition and secondly, by the identification of novel inhibitors. The cell-based screen employed cells expressing a fusion of the ATR-activating domain of TopBP1 with a fragment of the oestrogen receptor such that ATR was activated on exposure to tamoxifen. ATR activity was then measured by phosphorylation of histone H2AX by immunofluorescence. Using this screen NVP-BEZ235 (Fig. 3), which had previously been thought to be selective for PI-3K and mTOR, was demonstrated to be a potent inhibitor of ATR (IC_{50} = 100 nM) [86]. The most potent ATR inhibitor, ETP-46464 (IC_{50} = 25 nM) (Fig. 3), identified using the screen inhibited the restart of stalled replication forks and abrogated S-phase arrest after HU exposure.

Three novel small molecules, VE-821, AZ-20 and NU6027 (Fig. 3), have recently been identified as being ATR inhibitors [34, 87–89]. All compounds inhibited CHK1 phosphorylation at Ser^{345} but there were some subtle differences in their cytotoxicity. NU6027 (K_i = 100 nM in biochemical assays and IC_{50} = 6.7 μM in cell-based assays) enhanced the sensitivity of MCF7 cells to representatives of the major classes of DNA damaging agents: IR, DNA methylating agents (TMZ), antimetabolites (HU), topoisomerase I and II poisons (camptothecin and doxorubicin), and the DNA cross-linking agent, cisplatin, but not to the antitubulin agent, paclitaxel [34]. The specificity of NU6027 for ATR was confirmed using ATR-KD cells. In line with previous suggestions that TMZ and 6-thioguanine potentiation by ATR inactivation required an intact mismatch repair system [30, 46], NU6027 potentiation of TMZ was greater in MMR-defective cells than in the parental and MMR corrected

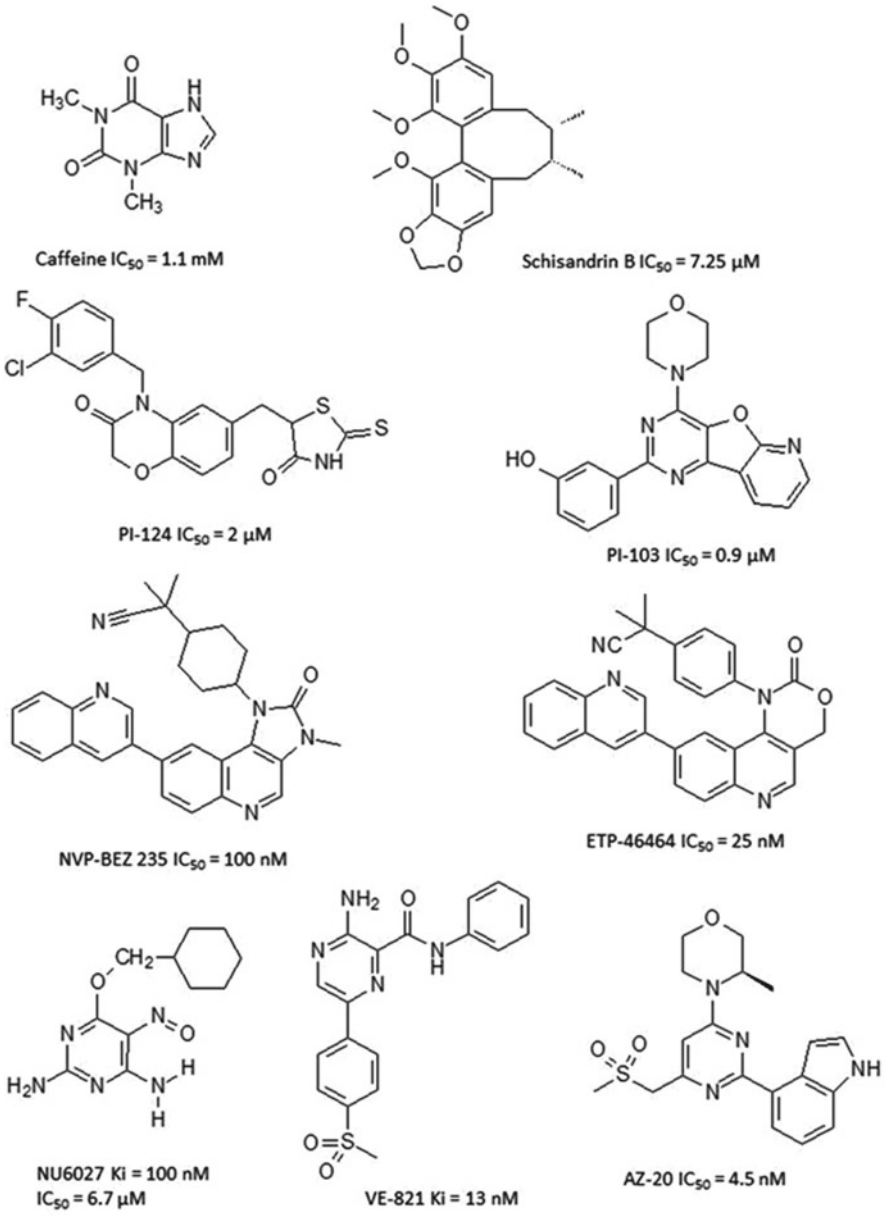

Fig. 3 Chemical structures of ATR inhibitors. Structurally diverse inhibitors of increasing potency have been identified since the prototype inhibitor, caffeine, was first described including the natural product Schisandrin B, pan PI-3K inhibitors (PI-103 and PI-124), those identified by high throughput screening (NVP-BEZ 235 and ETP-46464) and novel small molecule inhibitors (NU6027, VE-821 and AZ-20)

counterparts [34]. In the studies with NU6027 both p53 wt and mutant cell lines were potentiated with sensitization of TMZ being greatest in p53 mutant cells but cisplatin sensitization was greater in cells with functional p53.

VE-821 is a highly potent and specific ATR inhibitor (K_i = 13 nM), which enhanced the cytotoxicity of cisplatin, gemcitabine, camptothecin, etoposide and IR, but not docetaxel in HCT116 colon cancer cells. Cisplatin potentiation by VE-821 was much greater in a panel of human cancer cell lines in comparison to normal human cell lines and was more active in cells lacking p53 or ATM. In contrast to NU6027, VE-821 only caused a very modest sensitization of wild-type p53 MCF7 cells to cisplatin [88]. Recently, VE-821 has been investigated as a radiosensitiser in hypoxic cells. Regions of hypoxia develop in solid tumours because of inefficient tumour vasculature and this contributes to chemoresistance and radioresistance [90]. Not only did VE-821 enhance IR-induced cytotoxicity in a panel of 12 human cancer cell lines, but it also caused a more profound radiosensitisation in cells cultured in levels of hypoxia typically found in solid tumours. VE-821 also increased re-oxygenation-induced DNA damage and decreased the survival of cells undergoing re-oxygenation [91].

AZ-20 is reported to be an even more potent ATR inhibitor with an IC_{50} of 4.5 nM in biochemical assays and 51 nM in cellular assays. This inhibitor was active as a single agent both in vitro and in vivo, and at an oral dose of 25 mg/kg bid or 50 mg/kg qd; it inhibited the growth of LoVo xenografts [89]. This is the first report of an ATR inhibitor in an in vivo model and although only published in abstract form the full data on this compound are eagerly awaited.

1.9 Single Agent Activity and Potential Synthetic Lethalities

In addition to the single agent activity of AZ-20, described above, the synthetic lethality of ATR inhibitors has been investigated. Synthetic lethality is an exciting concept in cancer therapy; it is used to describe the phenomenon where inactivation (or dysregulation) of two complementary pathways results in cell death but inactivation of either alone does not compromise viability. If one of the pathways is already compromised in the cancer cell then targeting of the other pathway can result in tumour-selective cell kill. The concept was applied to cancer to explain the selective killing of cancer cells with particular molecular defects, by some agents over 15 years ago. More recently synthetic lethality by agents that are not cytotoxic in their own right has been demonstrated. Inhibitors of poly(ADP-ribose) polymerase, an enzyme that plays a critical role in the repair of DNA SSB by the BER pathway were profoundly cytotoxic to HRR-defective cancer cells but did not affect the viability of cells with functional HRR [92, 93]. As described above, ATR plays an important role in HRR and NU6027 inhibited RAD51 focus formation (indicative of HRR suppression), so it was a logical extension of this work to investigate ATR inhibition in cells with BER defects. NU6027 was more cytotoxic in cells lacking the BER scaffold protein, XRCC1, and also in the presence of a PARP inhibitor, suggesting the potential for synthetic lethality [34]. These findings are relevant to the molecular biology

of cancer because polymorphisms in XRCC1 and other defects in DNA single-strand break repair e.g. those due to aberration in DNA polβ are also associated with cancer [94, 95] and this may be exploitable by ATR inhibition. Other recent data demonstrate that caffeine selectively radiosensitises polβ defective cells [96] implicating that ATR inhibition would have broad applicability in cancer.

It is well recognised that oncogene activation and hyperactive growth factor signalling itself causes oncogenic stress, characterised by stalled/collapsed replication forks, making such cancer cells particularly dependent on the ATR pathway for survival [97]. It was exciting to discover that inactivation of ATR or CHK1 is synthetically lethal in oncogene-activated cancer cells. Knocking down ATR to 16% of normal levels was synthetically lethal in ras-transformed cells [98]. By analogy, inhibition of both CHK1/CHK2 with AZD7762 induced cell death and significantly delayed disease progression of Myc-over-expressing lymphoma cells in vivo [99, 100]. Cyclin E, which promotes S-phase entry, is commonly over-expressed in cancer and leads to replication stress and DNA damage. The ATR inhibitor, ETP-46464 was cytotoxic to cells with induced over-expression of cyclin E [86].

1.10 Differences Between ATR Inhibitors and CHK1 Inhibitors

A number of CHK1 inhibitors are undergoing clinical evaluation and it could be argued that ATR inhibitors will have a very similar profile. However, although CHK1 is thought to be the major target of ATR, ATR inhibitors are not necessarily the same as CHK1 inhibitors and key differences have been observed. For example the inhibition of cisplatin, carboplatin and oxaliplatin by ATR siRNA seen in a variety of cell lines was not replicated when CHK1 was inhibited [77]. DLD-1 cells expressing Seckel mutant ATR were much more sensitive to cisplatin and MMC than those expressing mutant CHK1; indeed the ATR mutant cells were generally more sensitive to a range of cytotoxic drugs showing the importance of other ATR substrates [101]. The effects of the CHK1 inhibitor, PF-00477736, was compared with those of NU6027: unlike NU6027, PF-00477736 caused only modest sensitization of cisplatin and camptothecin but did enhance the cytotoxicity of paclitaxel in MCF7 cells in line with previous studies with PF-00477736 [102]. Interestingly, PF-00477736 did not inhibit the formation of RAD51 foci and, instead, increased them, suggesting that PF-00477736 stimulates HRR rather than inhibiting it [34]. Clearly therefore inhibitors of ATR will have a different spectrum of activity compared to CHK1 inhibitors.

1.11 Pharmacodynamic Biomarkers of ATR Inhibition

Since ATR inhibitors are proposed to have minimal toxicity in normal cells MTD may not be the best endpoint for clinical trials with ATR inhibitors. Pharmacodynamic biomarkers of ATR inhibition are needed to guide these trials. One possibility is

CHK1 phosphorylation, which has been used in in vitro studies. However, it may be easier to use a general marker of DNA damage signalling, such as the phosphorylation of histone H2AX (γH2AX) by ATR. Although not specific for ATR, as ATM and DNA-PK also phosphorylate H2AX in response to DNA DSB and stalled replication forks, methods already exist to determine γH2AX nuclear foci, or levels by immunofluorescence microscopy, flow cytometry or immunoblotting.

1.12 Future Prospects

The accumulating preclinical data demonstrates that the inactivation of ATR, by genetic means or small molecule inhibitors, enhances the cytotoxicity of all classes of DNA damaging anticancer agents. Moreover, these data support the premise that sensitization is greatest in cells with dysfunctional G1 control that distinguishes tumour cells from normal tissues. This evidence indicates that ATR inhibitors will have broad application as chemo- and radiosensitisers with minimal toxicities. In the last few years a number of potent and chemically diverse small molecule inhibitors of ATR have been identified and it is to be hoped that these will enter clinical evaluation in the near future.

Perhaps the most exciting aspect is the potential for synthetic lethality of ATR inhibitors in cells lacking BER or undergoing replication stress due to oncogene activation, amplification of growth factor signalling or cell cycle pathways. More work is needed to confirm these initial findings and establish biomarkers for the determinants of sensitivity to ATR inhibitors.

References

1. Durocher D, Jackson SP (2001) DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme? Curr Opin Cell Biol 13(2):225–231
2. Matsuoka S et al (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science 316(5828):1160–1166
3. Bentley NJ et al (1996) The Schizosaccharomyces pombe rad3 checkpoint gene. EMBO J 15(23):6641–6651
4. Brown EJ, Baltimore D (2000) ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev 14(4):397–402
5. Schlegel R, Pardee AB (1986) Caffeine-induced uncoupling of mitosis from the completion of DNA replication in mammalian cells. Science 232(4755):1264–1266
6. O'Driscoll M et al (2003) A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. Nat Genet 33(4):497–501
7. Tauchi H et al (2002) Nijmegen breakage syndrome gene, NBS1, and molecular links to factors for genome stability. Oncogene 21(58):8967–8980
8. Casper AM et al (2004) Chromosomal instability at common fragile sites in Seckel syndrome. Am J Hum Genet 75(4):654–660
9. Cliby WA et al (1998) Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. EMBO J 17(1):159–169

10. Cliby WA et al (2002) S phase and G2 arrests induced by topoisomerase I poisons are dependent on ATR kinase function. J Biol Chem 277(2):1599–1606
11. Unsal-Kacmaz K et al (2002) Preferential binding of ATR protein to UV-damaged DNA. Proc Natl Acad Sci USA 99(10):6673–6678
12. Saleh-Gohari N et al (2005) Spontaneous homologous recombination is induced by collapsed replication forks that are caused by endogenous DNA single-strand breaks. Mol Cell Biol 25(16):7158–7169
13. Burhans WC, Weinberger M (2007) DNA replication stress, genome instability and aging. Nucleic Acids Res 35(22):7545–7556
14. Paulsen RD, Cimprich KA (2007) The ATR pathway: fine-tuning the fork. DNA Repair (Amst) 6(7):953–966
15. Lindahl T (1993) Instability and decay of the primary structure of DNA. Nature 362(6422): 709–715
16. Vilenchik MM, Knudson AG (2003) Endogenous DNA double-strand breaks: production, fidelity of repair, and induction of cancer. Proc Natl Acad Sci USA 100(22):12871–12876
17. Hang B (2010) Formation and repair of tobacco carcinogen-derived bulky DNA adducts. J Nucleic Acids 2010:709521
18. Hoeijmakers JH (2001) Genome maintenance mechanisms for preventing cancer. Nature 411(6835):366–374
19. Shiotani B, Zou L (2009) Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks. Mol Cell 33(5):547–558
20. Symington LS, Gautier J (2011) Double-strand break end resection and repair pathway choice. Annu Rev Genet 45:247–271
21. Morishima K et al (2007) TopBP1 associates with NBS1 and is involved in homologous recombination repair. Biochem Biophys Res Commun 362(4):872–879
22. Delacroix S et al (2007) The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1. Genes Dev 21(12):1472–1477
23. Dart DA et al (2004) Recruitment of the cell cycle checkpoint kinase ATR to chromatin during S-phase. J Biol Chem 279(16):16433–16440
24. Zou L, Elledge SJ (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science 300(5625):1542–1548
25. Itakura E et al (2004) ATR-dependent phosphorylation of ATRIP in response to genotoxic stress. Biochem Biophys Res Commun 323(4):1197–1202
26. Zou L, Liu D, Elledge SJ (2003) Replication protein A-mediated recruitment and activation of Rad17 complexes. Proc Natl Acad Sci USA 100(24):13827–13832
27. Yoshioka K, Yoshioka Y, Hsieh P (2006) ATR kinase activation mediated by MutSalpha and MutLalpha in response to cytotoxic O6-methylguanine adducts. Mol Cell 22(4):501–510
28. Wang Y, Qin J (2003) MSH2 and ATR form a signaling module and regulate two branches of the damage response to DNA methylation. Proc Natl Acad Sci USA 100(26):15387–15392
29. Liu Y et al (2010) Interactions of human mismatch repair proteins MutSalpha and MutLalpha with proteins of the ATR-Chk1 pathway. J Biol Chem 285(8):5974–5982
30. Caporali S et al (2004) DNA damage induced by temozolomide signals to both ATM and ATR: role of the mismatch repair system. Mol Pharmacol 66(3):478–491
31. Wang X et al (2006) Rad17 phosphorylation is required for claspin recruitment and Chk1 activation in response to replication stress. Mol Cell 23(3):331–341
32. Takai H et al (2000) Aberrant cell cycle checkpoint function and early embryonic death in Chk1(−/−) mice. Genes Dev 14(12):1439–1447
33. Walker M et al (2009) Chk1 C-terminal regulatory phosphorylation mediates checkpoint activation by de-repression of Chk1 catalytic activity. Oncogene 28(24):2314–2323
34. Peasland A et al (2011) Identification and evaluation of a potent novel ATR inhibitor, NU6027, in breast and ovarian cancer cell lines. Br J Cancer 105(3):372–381
35. Liu Q et al (2000) Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev 14(12):1448–1459

36. Parsels LA et al (2011) Assessment of chk1 phosphorylation as a pharmacodynamic biomarker of chk1 inhibition. Clin Cancer Res 17(11):3706–3715
37. Sorensen CS, Syljuasen RG (2012) Safeguarding genome integrity: the checkpoint kinases ATR, CHK1 and WEE1 restrain CDK activity during normal DNA replication. Nucleic Acids Res 40(2):477–486
38. Chen MS, Ryan CE, Piwnica-Worms H (2003) Chk1 kinase negatively regulates mitotic function of Cdc25A phosphatase through 14-3-3 binding. Mol Cell Biol 23(21):7488–7497
39. Dai Y, Grant S (2010) New insights into checkpoint kinase 1 in the DNA damage response signaling network. Clin Cancer Res 16(2):376–383
40. Lee J, Kumagai A, Dunphy WG (2001) Positive regulation of Wee1 by Chk1 and 14-3-3 proteins. Mol Biol Cell 12(3):551–563
41. Flynn RL, Zou L (2011) ATR: a master conductor of cellular responses to DNA replication stress. Trends Biochem Sci 36(3):133–140
42. Casper AM et al (2002) ATR regulates fragile site stability. Cell 111(6):779–789
43. Petermann E, Caldecott KW (2006) Evidence that the ATR/Chk1 pathway maintains normal replication fork progression during unperturbed S phase. Cell Cycle 5(19):2203–2209
44. Yajima H, Lee KJ, Chen BP (2006) ATR-dependent phosphorylation of DNA-dependent protein kinase catalytic subunit in response to UV-induced replication stress. Mol Cell Biol 26(20):7520–7528
45. Shigechi T et al (2012) ATR-ATRIP kinase complex triggers activation of the Fanconi anemia DNA repair pathway. Cancer Res 72(5):1149–1156
46. Yamane K, Taylor K, Kinsella TJ (2004) Mismatch repair-mediated G2/M arrest by 6-thioguanine involves the ATR-Chk1 pathway. Biochem Biophys Res Commun 318(1):297–302
47. Stauffer D et al (2007) p300/CREB-binding protein interacts with ATR and is required for the DNA replication checkpoint. J Biol Chem 282(13):9678–9687
48. Chen G et al (1999) Radiation-induced assembly of Rad51 and Rad52 recombination complex requires ATM and c-Abl. J Biol Chem 274(18):12748–12752
49. Di Virgilio M, Ying CY, Gautier J (2009) PIKK-dependent phosphorylation of Mre11 induces MRN complex inactivation by disassembly from chromatin. DNA Repair (Amst) 8(11): 1311–1320
50. Bolderson E et al (2010) Phosphorylation of Exo1 modulates homologous recombination repair of DNA double-strand breaks. Nucleic Acids Res 38(6):1821–1831
51. Cortez D et al (1999) Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science 286(5442):1162–1166
52. Zhang F et al (2009) PALB2 links BRCA1 and BRCA2 in the DNA-damage response. Curr Biol 19(6):524–529
53. Jensen RB, Carreira A, Kowalczykowski SC (2010) Purified human BRCA2 stimulates RAD51-mediated recombination. Nature 467(7316):678–683
54. Liu J et al (2010) Human BRCA2 protein promotes RAD51 filament formation on RPA-covered single-stranded DNA. Nat Struct Mol Biol 17(10):1260–1262
55. Thorslund T et al (2010) The breast cancer tumor suppressor BRCA2 promotes the specific targeting of RAD51 to single-stranded DNA. Nat Struct Mol Biol 17(10):1263–1265
56. Wang H et al (2004) ATR affecting cell radiosensitivity is dependent on homologous recombination repair but independent of nonhomologous end joining. Cancer Res 64(19): 7139–7143
57. Sorensen CS et al (2005) The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. Nat Cell Biol 7(2):195–201
58. Ward IM, Chen J (2001) Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. J Biol Chem 276(51):47759–47762
59. Paull TT et al (2000) A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol 10(15):886–895
60. Chu WK et al (2010) BLM has early and late functions in homologous recombination repair in mouse embryonic stem cells. Oncogene 29(33):4705–4714

61. Davies SL et al (2004) Phosphorylation of the Bloom's syndrome helicase and its role in recovery from S-phase arrest. Mol Cell Biol 24(3):1279–1291
62. Otterlei M et al (2006) Werner syndrome protein participates in a complex with RAD51, RAD54, RAD54B and ATR in response to ICL-induced replication arrest. J Cell Sci 119(Pt 24):5137–5146
63. Tripathi V, Kaur S, Sengupta S (2008) Phosphorylation-dependent interactions of BLM and 53BP1 are required for their anti-recombinogenic roles during homologous recombination. Carcinogenesis 29(1):52–61
64. Patro BS et al (2011) WRN helicase regulates the ATR-CHK1-induced S-phase checkpoint pathway in response to topoisomerase-I-DNA covalent complexes. J Cell Sci 124(Pt 23): 3967–3979
65. Vasquez KM (2010) Targeting and processing of site-specific DNA interstrand crosslinks. Environ Mol Mutagen 51(6):527–539
66. D'Andrea AD (2010) Susceptibility pathways in Fanconi's anemia and breast cancer. N Engl J Med 362(20):1909–1919
67. Deans AJ, West SC (2011) DNA interstrand crosslink repair and cancer. Nat Rev Cancer 11(7):467–480
68. Massague J (2004) G1 cell-cycle control and cancer. Nature 432(7015):298–306
69. Storz P (2005) Reactive oxygen species in tumor progression. Front Biosci 10:1881–1896
70. Babior BM (1999) NADPH oxidase: an update. Blood 93(5):1464–1476
71. Berasain C et al (2009) Inflammation and liver cancer: new molecular links. Ann N Y Acad Sci 1155:206–221
72. Wiseman H, Halliwell B (1996) Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. Biochem J 313(Pt 1):17–29
73. Garrett MD, Collins I (2011) Anticancer therapy with checkpoint inhibitors: what, where and when? Trends Pharmacol Sci 32(5):308–316
74. Wagner JM, Kaufmann SH (2010) Prospects for the Use of ATR Inhibitors to Treat Cancer. Pharmaceuticals 3(5):1311–1334
75. Nghiem P et al (2001) ATR inhibition selectively sensitizes G1 checkpoint-deficient cells to lethal premature chromatin condensation. Proc Natl Acad Sci USA 98(16):9092–9097
76. Wilsker D, Bunz F (2007) Loss of ataxia telangiectasia mutated- and Rad3-related function potentiates the effects of chemotherapeutic drugs on cancer cell survival. Mol Cancer Ther 6(4):1406–1413
77. Wagner JM, Karnitz LM (2009) Cisplatin-induced DNA damage activates replication checkpoint signaling components that differentially affect tumor cell survival. Mol Pharmacol 76(1):208–214
78. Caporali S et al (2008) AKT is activated in an ataxia-telangiectasia and Rad3-related-dependent manner in response to temozolomide and confers protection against drug-induced cell growth inhibition. Mol Pharmacol 74(1):173–183
79. Flatten K et al (2005) The role of checkpoint kinase 1 in sensitivity to topoisomerase I poisons. J Biol Chem 280(14):14349–14355
80. Powell SN et al (1995) Differential sensitivity of p53(−) and p53(+) cells to caffeine-induced radiosensitization and override of G2 delay. Cancer Res 55(8):1643–1648
81. Yao SL et al (1996) Selective radiosensitization of p53-deficient cells by caffeine-mediated activation of p34cdc2 kinase. Nat Med 2(10):1140–1143
82. Sarkaria JN et al (1999) Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res 59(17):4375–4382
83. Chalmers AJ et al (2009) Cytotoxic effects of temozolomide and radiation are additive- and schedule-dependent. Int J Radiat Oncol Biol Phys 75(5):1511–1519
84. Nishida H et al (2009) Inhibition of ATR protein kinase activity by schisandrin B in DNA damage response. Nucleic Acids Res 37(17):5678–5689
85. Knight ZA et al (2006) A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. Cell 125(4):733–747
86. Toledo LI et al (2011) A cell-based screen identifies ATR inhibitors with synthetic lethal properties for cancer-associated mutations. Nat Struct Mol Biol 18(6):721–727

87. Charrier JD et al (2011) Discovery of potent and selective inhibitors of ataxia telangiectasia mutated and Rad3 related (ATR) protein kinase as potential anticancer agents. J Med Chem 54(7):2320–2330
88. Reaper PM et al (2011) Selective killing of ATM- or p53-deficient cancer cells through inhibition of ATR. Nat Chem Biol 7(7):428–430
89. Jacq X et al. (2012) *AZ20,* a novel potent and selective inhibitor of ATR kinase with in vivo antitumour activity. Cancer Res. 72(8) Suppl1 Abs#1823
90. Graeber TG et al (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 379(6560):88–91
91. Pires IM et al (2012) Targeting radiation-resistant hypoxic tumour cells through ATR inhibition. Br J Cancer 107(2):291–299
92. Bryant HE et al (2005) Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature 434(7035):913–917
93. Farmer H et al (2005) Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 434(7035):917–921
94. Starcevic D, Dalal S, Sweasy JB (2004) Is there a link between DNA polymerase beta and cancer? Cell Cycle 3(8):998–1001
95. Kiyohara C, Takayama K, Nakanishi Y (2006) Association of genetic polymorphisms in the base excision repair pathway with lung cancer risk: a meta-analysis. Lung Cancer 54(3):267–283
96. Neijenhuis S et al (2010) Targeted radiosensitization of cells expressing truncated DNA polymerase {beta}. Cancer Res 70(21):8706–8714
97. Halazonetis TD, Gorgoulis VG, Bartek J (2008) An oncogene-induced DNA damage model for cancer development. Science 319(5868):1352–1355
98. Gilad O et al (2010) Combining ATR suppression with oncogenic Ras synergistically increases genomic instability, causing synthetic lethality or tumorigenesis in a dosage-dependent manner. Cancer Res 70(23):9693–9702
99. Hoglund A et al (2011) Therapeutic implications for the induced levels of Chk1 in Myc-expressing cancer cells. Clin Cancer Res 17(22):7067–7079
100. Ferrao PT et al (2012) Efficacy of CHK inhibitors as single agents in MYC-driven lymphoma cells. Oncogene 31(13):1661–1672
101. Wilsker D et al (2012) Targeted mutations in the ATR pathway define agent-specific requirements for cancer cell growth and survival. Mol Cancer Ther 11(1):98–107
102. Zhang C et al (2009) PF-00477736 mediates checkpoint kinase 1 signaling pathway and potentiates docetaxel-induced efficacy in xenografts. Clin Cancer Res 15(14):4630–4640