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ATM and ATR as therapeutic targets in cancer

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ABSTRACT

In order to maintain genomic stability, cells have developed sophisticated signalling pathways to enable DNA damage or DNA replication stress to be resolved. Key mediators of this DNA damage response (DDR) are the ATM and ATR kinases, which induce cell cycle arrest and facilitate DNA repair via their downstream targets. Inhibiting the DDR has become an attractive therapeutic concept in cancer therapy, since (i) resistance to genotoxic therapies has been associated with increased DDR signalling, and (ii) many cancers have defects in certain components of the DDR rendering them highly dependent on the remaining DDR pathways for survival. ATM and ATR act as the apical regulators of the response to DNA double strand breaks and replication stress, respectively, with overlapping but non-redundant activities. Highly selective small molecule inhibitors of ATM and ATR are currently in preclinical and clinical development, respectively. Preclinical data have provided a strong rationale for clinical testing of these compounds both in combination with radio- or chemotherapy, and in synthetic lethal approaches to treat tumours with deficiencies in certain DDR components. Whole genome sequencing studies have reported that mutations in DDR genes occur with a high frequency in many common tumour types, suggesting that a synthetic lethal approach with ATM or ATR inhibitors could have widespread utility, providing that appropriate biomarkers are developed.

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Abbreviations: A-T, ataxia–telangiectasia; ATM, ataxia–telangiectasia mutated; ATR, ataxia–telangiectasia and Rad3 related; CHK1, checkpoint kinase 1; CHK2, checkpoint kinase 2; DDR, DNA damage response; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, double-strand break; FAT, FRAP–ATM–TRRAP; FATC, FAT C-terminal; IR, ionising radiation; kd, kinase-dead; MRN, MRE11–RAD50–NBS1; PI3K, phosphatidylinositol 3-kinase; PIKK, phosphatidylinositol 3-kinase-related kinase; PARP, poly(ADP-ribose)-polymerase; RPA, replication protein A; ssDNA, single-stranded DNA.

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1. Introduction: the DNA damage response – important implications for tumour development and treatment

Cells are invariably challenged by tens of thousands of lesions inflicted on their DNA everyday (Lindahl, 1993). This DNA damage can be caused exogenously by exposure to different types of radiation or genotoxic agents, or endogenously through, for example, base depurination and deamination or reactive by-products of cellular metabolism (Lindahl & Barnes, 2000). If unrepaired or aberrantly repaired, such lesions may

prove lethal to the cell or give rise to deleterious mutations that can affect cell viability or induce aberrant cellular behaviour leading to the development of malignancies such as cancer. Because genetic alterations have such a significant potential impact on survival and viability of a cell, as well as the organism as a whole, cells have developed a complex network of signalling pathways – collectively known as the DNA damage response (DDR) – that orchestrates the detection and repair of DNA damage with transient cell cycle arrest to ensure maintenance of genomic stability and cell viability (Jackson & Bartek, 2010; Ciccia & Elledge, 2011).

The DDR plays an important role in many aspects of cancer. First, deficiencies in DDR mechanisms have been shown to be contributing factors in many stages of tumour development. Numerous hereditary cancer predispositions result from mutations in DNA repair genes (Goode et al., 2002; Negrini et al., 2010). For example, women who carry a defective allele of the BRCA1 or BRCA2 genes, which encode for two proteins centrally involved in the repair of DNA double strand breaks (DSBs) by homologous recombination, are at much higher risk of developing breast and ovarian cancers than women in the general population (Pal et al., 2005; Levy-Lahad & Friedman, 2007). Furthermore, several studies have demonstrated activation of DDR proteins during early stages of tumorigenesis (Bartkova et al., 2005; Gorgoulis et al., 2005). This response has been linked to the induction of replication stress and DNA damage, caused by abnormal replication due to aberrant oncogene activation (Bartkova et al., 2006; Di Micco et al., 2006). It has been suggested that this activation of DDR pathways may limit tumour development in its early stages by acting as a barrier for proliferation of aberrant cells (Bartkova et al., 2005; Gorgoulis et al., 2005), particularly through activation of the tumour suppressor protein p53 (Halazonetis et al., 2008). Therefore, it is unsurprising that many malignant tumours show functional loss or deregulation of key proteins involved in the DDR and cell cycle regulation, most notably p53 and ATM (Cancer Genome Atlas Research Network, 2012a, 2012b, 2012c, 2014; Kandoth et al., 2013), MRE11 (Ottini et al., 2004; Bartkova et al., 2008; Choudhury et al., 2010), BRCA1/2 (Cancer Genome Atlas Research Network, 2012c; Kandoth et al., 2013) or SMC1 (Kandoth et al., 2013). Such mutations may allow pre-cancerous cells to breach the proliferation barrier posed by the DDR, thereby allowing the progression of pre-malignant lesions to malignant carcinomas. In addition, deregulation of DDR pathways also contributes to the development of genomic instability, a characteristic of human cancers that accelerates the genetic alterations which drive tumour development (Negrini et al., 2010; Hanahan & Weinberg, 2011).

DDR mechanisms are also relevant to the effectiveness of standard cancer treatments, such as radio- and chemotherapy. These treatments rely on the induction of DNA damage, which is particularly cytotoxic for proliferating cells and hence, very effective in targeting highly proliferative cancer cells. Cancer cells can, however, resist the lethal effects of genotoxic cancer therapy by activating the DDR (Karnitz et al., 2005; Myers et al., 2009; Woods & Turchi, 2013), and since chemo- and radiotherapy generally lack selectivity towards cancer cells, the toxicity induced in normal tissues and the resulting side effects are limiting factors for the dose and duration of therapy. This is one of the reasons why these therapies, though effective, often fail to be curative. Also, tumours can develop resistance to radio- or chemotherapy allowing tumour recurrence following an initial response to therapy. Several studies have shown, that the development of resistance to different types of genotoxic therapy can be caused by deregulation and over-expression of different components of the DNA damage response (Bao et al., 2006; Oliver et al., 2010; Bobola et al., 2012).

Regulators of the DDR have therefore become attractive targets for cancer therapy primarily through two potential approaches. First, to be used as chemo- or radiosensitisers to increase the effectiveness of standard genotoxic treatments and to help prevent or overcome the development of resistance. Second, to exploit defects in DDR mechanisms as potentially targetable weaknesses through synthetic lethal

approaches. While defects in DDR components may, on the one hand, give cancer cells a growth advantage, allowing them to survive and proliferate despite oncogene-induced replication stress and genomic instability, they may also drive a reliance of cancer cells on any remaining DDR pathways in order to survive DNA damage. Targeting of such remaining pathways may therefore be selectively toxic to cancer cells with mutations in certain DDR genes. The potential of this approach was first demonstrated in cells harbouring mutations in the breast and ovarian cancer susceptibility genes BRCA1 and BRCA2, which were shown to be highly sensitive to small molecule inhibitors of poly(ADP-ribose)-polymerase (PARP), a DDR protein that is involved in the detection and repair of DNA single strand breaks by base excision repair (Bryant et al., 2005; Farmer et al., 2005). The PARP inhibitor olaparib has since shown promise for the treatment of BRCA1/2 mutated breast or ovarian cancer in clinical trials (Fong et al., 2009; Audeh et al., 2010; Tutt et al., 2010). Further studies have demonstrated that inhibition of other components of the DDR machinery can sensitise cancer cells to DNA damaging treatments, including DNA-PKcs (Zhao et al., 2006), ataxia-telangiectasia mutated (ATM) (Rainey et al., 2008; Golding et al., 2012), ataxia-telangiectasia and Rad3 related (ATR) (Fokas et al., 2012; Pires et al., 2012; Prevo et al., 2012; Huntoon et al., 2013), or their downstream targets CHK1 and CHK2 (Matthews et al., 2007; Blasina et al., 2008; Mitchell et al., 2010; Riesterer et al., 2011).

Here, we focus on the role of ATR and ATM in the DNA damage response and the current status of development of specific inhibitors targeting these pathways. We review the studies elucidating the potential of these inhibitors to act as radio- and chemosensitisers and discuss the genetic alterations or cellular phenotypes that suggest which patient subsets may benefit most from these therapies. We also give examples of synthetic lethal approaches that target these two DDR kinases.

2. The phosphatidylinositol-3 kinase-related kinase (PIKK) family

ATM and ATR are members of the phosphatidylinositol 3-kinase-related kinase (PIKK) family of serine/threonine protein kinases, which also comprises DNA-dependent protein kinase catalytic subunit (DNA-PKcs/PRKDC), mammalian target of rapamycin (MTOR/FRAP) and suppressor of morphogenesis in genitalia (SMG1). The cellular functions of these protein kinases range from regulation of the DNA damage response (DDR) to cell survival, proliferation, metabolism, differentiation, motility and nonsense-mediated mRNA decay (Lempiäinen & Halazonetis, 2009; Jackson & Bartek, 2010; Ciccia & Elledge, 2011). A sixth member of the PIKK family, transformation/transcription associated protein (TRRAP), serves as component of various histone acetyltransferase complexes and plays a role in the epigenetic regulation of transcription, but possesses no kinase activity (Lempiäinen & Halazonetis, 2009).

The members of the PIKK kinase family show considerable similarities in their domain architecture and extensive sequence homology, particularly in their C-terminal kinase domain and the flanking FAT (FRAP-ATM-TRRAP) and FATC (FAT C-terminal) domains (Fig. 1) (Keith & Schreiber, 1995; Bosotti et al., 2000). Even though the functions of the FAT and FATC domains are not yet fully understood, both domains have been implicated in the regulation of kinase activity. The N-terminal region is poorly conserved between PIKK family members and is believed to be important for the interaction with various substrates and adapter proteins (Fernandes et al., 2005).

In ATR, the N-terminus also contains the binding site for ATRIP (ATR-interacting protein), which regulates the localisation of ATR to sites of replication stress and DNA damage and is essential for ATR signalling (Cortez et al., 2001; Zou & Elledge, 2003).

Many reports have linked members of this kinase family to tumorigenesis and survival of cancer cells following therapy, rendering these kinases attractive potential targets for cancer treatment. However, the development of selective inhibitors has not been without difficulty, due to the high degree of homology in the kinase domain, increasing the likelihood of inhibition of other PIKK family members, which may

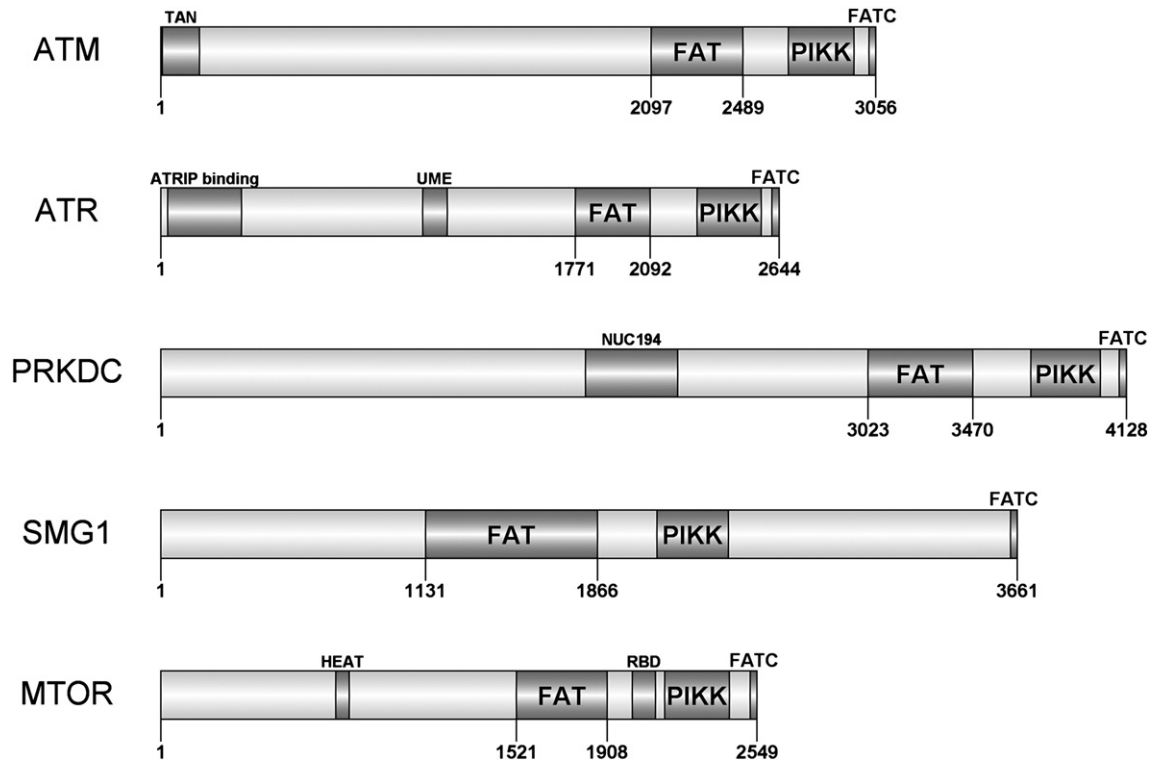


Fig. 1. Schematic diagrams of the domain structure of phosphatidylinositol 3-kinase-related kinases (PIKKs). Known structural domains are shown for each protein. ATM (ataxia–telangiectasia mutated), ATR (ataxia–telangiectasia and Rad3 related), PRKDC (DNA-dependent protein kinase catalytic subunit; DNA-PKcs), SMG1 (suppressor of morphogenesis in genitalia), MTOR (mammalian target of rapamycin), FAT (FRAP–ATM–TRRAP domain), FATC (FAT C-terminal domain), PIKK (phosphatidylinositol 3-kinase-related kinase domain), TAN (Tel1/ATM N-terminal motif), ATRIP (ATR interacting protein), UME (UVSB PI3 kinase, MEI-41 and ESR1 domain), NUC194 (domain B in the catalytic subunit of DNA-dependent protein kinase), RBD (rapamycin binding domain), HEAT (Huntingtin, elongation factor 3, protein phosphatase 2A, and the yeast kinase TOR1-like repeat).

increase toxicity. NVP-BE235 is an example for a compound with such cross-reactivity, which will be discussed later.

3. Ataxia–telangiectasia mutated activation and downstream signalling

Of the many different types of DNA lesions, DNA double strand breaks (DSBs) are amongst the most deleterious. It has been suggested that a single unrepaired DSB may be sufficient to induce cell death (Bennett et al., 1993), whereas misrepaired DSBs can result in loss of genetic information, potentially harmful mutations or chromosomal rearrangements, which can lead to cancer development.

ATM is one of the central kinases involved in the cellular response to DNA DSBs which may arise, for example, intrinsically through the collapse of stalled replication forks or extrinsically through exposure to ionising radiation (IR) (van Gent et al., 2001). Despite great advancement in our understanding of ATM signalling and function in recent years, the complex mechanisms involved in its activation are not yet fully resolved.

In its inactive state, ATM forms homodimers or higher order multimers which dissociate into active monomers following rapid intermolecular autophosphorylation of serine 1981 upon ATM activation (Bakkenist & Kastan, 2003). Since the initial discovery of this autophosphorylation site and its role in ATM activation, other ATM post-translational modifications have been reported, including additional phosphorylation sites and an acetylation site, which regulate ATM activity (Kozlov et al., 2006; Sun et al., 2007). The recruitment of ATM to sites of DNA DSBs is mediated via the MRE11–RAD50–NBS1 (MRN) complex (Lee & Paull, 2005). The MRN complex quickly assembles at sites of DNA DSBs, where it acts as a damage sensor that can also form a physical bridge spanning the DSB (Stracker & Petrini, 2011). ATM recruitment has been shown to require its binding

to the C-terminus of NBS1, an interaction that also enhances the kinase activity of ATM (You et al., 2005). Immediately following its recruitment to sites of DNA DSBs, ATM contributes to the phosphorylation of the histone variant H2AX on Serine 139 (referred to as γ H2AX) (Burma et al., 2001). H2AX phosphorylation in turn initiates a cascade which assembles DDR components at the breakage site (Paull et al., 2000; Scully & Xie, 2013). Interestingly, MRN complex components not only modulate the activity of ATM, but are also amongst its downstream targets (Lim et al., 2000; Di Virgilio et al., 2009; Gatei et al., 2011, 2000). This suggests that ATM and the MRN complex work together at the sites of DNA DSBs to fine-tune the recruitment and dissociation of DDR factors and promote effective DNA damage repair.

ATM plays a crucial role in the activation of the G1/S cell cycle checkpoint, which prevents cells with damaged DNA from entering S-phase. This response is primarily mediated through the tumour suppressor protein p53, which was one of the first ATM downstream targets to be reported. In response to the induction of DNA DSBs, ATM directly phosphorylates p53 on serine 15 (Kastan et al., 1992; Siliciano et al., 1997; Banin et al., 1998; Canman et al., 1998). Checkpoint kinase 2 (CHK2) a key downstream target of ATM (Matsuoka et al., 1998) and mediator of ATM signalling also phosphorylates p53, on serine 20 (Chehab et al., 1999, 2000). This phosphorylation of p53 leads to its stabilisation by preventing its Mdm2-mediated ubiquitinylation and degradation (Haupt et al., 1997; Chehab et al., 1999; Marine & Lozano, 2010). ATM further contributes to the accumulation and stabilisation of p53 by directly phosphorylating Mdm2 (Khosravi et al., 1999). Upon activation and accumulation in the nucleus, p53 acts as transcription factor and drives the expression of genes involved in cell cycle checkpoint activation, such as p21, but also several genes which are involved in the induction of apoptosis (Sullivan et al., 2012). In addition to its role in the G1/S checkpoint, ATM also contributes to the activation of an

intra-S-phase checkpoint, as cells deficient in ATM do not reduce DNA synthesis following induction of DNA DSBs, a phenotype referred to as radioresistant DNA synthesis (Houldsworth & Lavin, 1980; Falck et al., 2001). The S-phase checkpoint functions of ATM in response to IR are partly mediated through phosphorylation of NBS1 and SMC1, a component of the cohesion complex (Lim et al., 2000; Kitagawa et al., 2004). Additional enforcement of the intra-S-phase checkpoint by ATM is mediated through its activation of CHK2, which induces ubiquitinylation and degradation of the S-phase-promoting phosphatase Cdc25A (Falck et al., 2001). Cdc25A promotes S-phase progression through activation of the cyclin-dependent kinase 2 (Cdk2) that is needed for DNA synthesis.

Recent studies have demonstrated that in response to IR, hundreds of substrates are phosphorylated in an ATM-dependent manner, clearly demonstrating the complexity of the ATM-mediated DDR pathways (Matsuoka et al., 2007; Bennetzen et al., 2010; Bensimon et al., 2010). However, evidence suggesting that DDR-independent roles of ATM is also emerging. It has been shown that ATM functions in the regulation of signalling pathways involved in maintaining cellular homeostasis, including cellular metabolism, responses to hypoxia and oxidative stress (Ditch & Paull, 2012).

4. Ataxia–telangiectasia and Rad3 related activation and downstream signalling

Like ATM, ATR is one of the central kinases involved in the DDR. ATR is activated by single stranded DNA structures, which may for example arise at resected DNA DSBs or stalled replication forks. When DNA polymerases stall during DNA replication, the replicative helicases continue to unwind the DNA ahead of the replication fork, leading to the generation of long stretches of single stranded DNA (ssDNA), which are then bound by the single-strand binding protein complex RPA (Replication protein A) (Wold, 1997; Byun et al., 2005). The recruitment of ATR/ATRIP complexes to these sites of replication stress and DNA damage is mediated by direct interaction of ATRIP with ssDNA-bound RPA (Zou & Elledge, 2003). Furthermore, RPA–ssDNA complexes stimulate the binding of the RAD17/RFC2–5 clamp loader complex to the damage sites. The presence of a dsDNA–ssDNA junction activates this complex to load the RAD9–HUS1–RAD1 (9–1–1) heterotrimer onto the DNA ends (Ellison & Stillman, 2001). The 9–1–1 complex in turn recruits TopBP1 which activates ATR (Kumagai et al., 2006; Delacroix et al., 2007; Lee et al., 2007).

Once activated, ATR acts via its downstream targets to promote DNA repair, stabilisation and restart of stalled replication forks and transient cell cycle arrest (Chen, 2000; Tibbetts et al., 2000; Sørensen et al., 2003; Xiao et al., 2003; Cimprich & Cortez, 2008; Dai & Grant, 2010; Errico & Costanzo, 2012). Many of these functions are mediated through the ATR downstream target CHK1. ATR plays an important role in the enforcement of the Intra-S-phase cell cycle checkpoint during normal S-phase progression and in response to DNA damage. It inhibits the firing of replication origins via mediating the degradation of Cdc25A through CHK1, which in turn slows the progression of DNA replication and provides time for resolution of the stress source (Sørensen et al., 2003; Xiao et al., 2003; Bartek et al., 2004). ATR is also a principal mediator of the G2/M cell cycle checkpoint to prevent the premature entry of cells into mitosis, before DNA replication is completed or in the presence of DNA damage. This ATR dependent G2/M cell cycle arrest is primarily mediated through two mechanisms: (i) the degradation of Cdc25A (Zhao et al., 2002; Xiao et al., 2003) and (ii) the phosphorylation of the Cdc25C phosphatase on serine 216 by CHK1, which creates a binding site for 14-3-3 proteins (Peng et al., 1997; Sanchez et al., 1997). The binding of Cdc25C to 14-3-3 proteins facilitates its export from the nucleus and cytoplasmic sequestration, thereby inhibiting its ability to dephosphorylate and activate nuclear Cdc2, which in turn prevents entry into mitosis (Kumagai & Dunphy, 1999; Graves et al., 2001).

5. Interplay between the ataxia–telangiectasia mutated and ataxia–telangiectasia and Rad3 related signalling pathways

Although ATM and ATR are activated by different types of DNA damage and act in distinct pathways, their downstream targets and the mediated responses are partially overlapping and dependent on the type of genotoxic stress (Helt et al., 2005). Both kinases share substrate specificity, that is they preferentially phosphorylate serine or threonine residues followed by glutamine (SQ/TQ motif) (Kim et al., 1999; O'Neill et al., 2000; Matsuoka et al., 2007). A large-scale proteomic study analysing proteins phosphorylated on consensus sites recognized by ATM and ATR in response to DNA damage identified over 700 putative targets (Matsuoka et al., 2007). Several of those targets, like p53 and the histone variant H2AX have been shown to be common targets of both kinases (Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999; Burma et al., 2001; Ward & Chen, 2001; Friesner et al., 2005). Even CHK1, which is often considered to be the most specific ATR downstream target, can be phosphorylated by ATM in response to IR on both Ser317 and Ser345 (Gatei et al., 2003; Sørensen et al., 2003; Helt et al., 2005). Through these common downstream targets, ATM and ATR cooperate in mediating the cellular responses to many genotoxic stresses and are together responsible for the maintenance of genomic stability by coordinating cell cycle progression with DNA repair (Abraham, 2001; Shiloh, 2003; Cimprich & Cortez, 2008).

ATM is generally regarded to be the principal mediator of the G1 cell cycle checkpoint, whereas the induction of the intra-S-phase and G2/M checkpoints are usually primarily related to ATR function. However, several studies have demonstrated that, depending on the cellular context and type of DNA damage, ATM also contributes to the activation and maintenance of the intra-S-phase and G2/M cell cycle arrest, suggesting a functional overlap of ATM and ATR signalling in checkpoint activation (Abraham, 2001; Shiloh, 2001). One example is the abovementioned enforcement of the intra-S-phase checkpoint, where both ATM and ATR can target the Cdc25A phosphatase for ubiquitin-dependent degradation thereby regulating the timing of replication origin firing in response to DNA damage (Falck et al., 2001; Xiao et al., 2003; Bartek et al., 2004). This suggests that both ATM and ATR are required for effective activation of the intra-S-phase checkpoint following DNA damage (see Fig. 2). ATM has also been demonstrated to mediate phosphorylation of Cdc25C via CHK2, thereby contributing to the activation of the G2/M cell cycle checkpoint (Matsuoka et al., 1998; Shiloh, 2001; Shiloh, 2003). In agreement with this, Thanasoula et al. have recently demonstrated that both ATM and ATR play a role in preventing telomere dysfunction-driven genomic instability through blocking mitotic entry with uncapped telomeres via degradation of the Cdc25C phosphatase (Thanasoula et al., 2012). Furthermore, studies in *Drosophila* and fission yeast have implicated that ATM and ATR control partially redundant pathways for telomere maintenance (Bi et al., 2005; Subramanian & Nakamura, 2010). A functional interplay between ATM and ATR in the maintenance of fragile site stability has also been suggested (Ozeri-Galai et al., 2008).

In conclusion, even though the ATM and ATR signalling pathways are not overall redundant, they are partially overlapping. Defects in one pathway may therefore, at least in part, be compensated for by the respective other pathway.

6. Ataxia–telangiectasia mutated as a therapeutic target

ATM is a known tumour suppressor which is frequently mutated in a broad range of human cancers including lung (Cancer Genome Atlas Research Network, 2012b, 2014), colorectal (Cancer Genome Atlas Research Network, 2012a), breast (Cancer Genome Atlas Research Network, 2012c) and haematopoietic cancers (Beà et al., 2013; Landau & Wu, 2013).

The initial scientific interest in ATM was, however, not focused on its role in tumour development. ATM was first described in 1995 as the

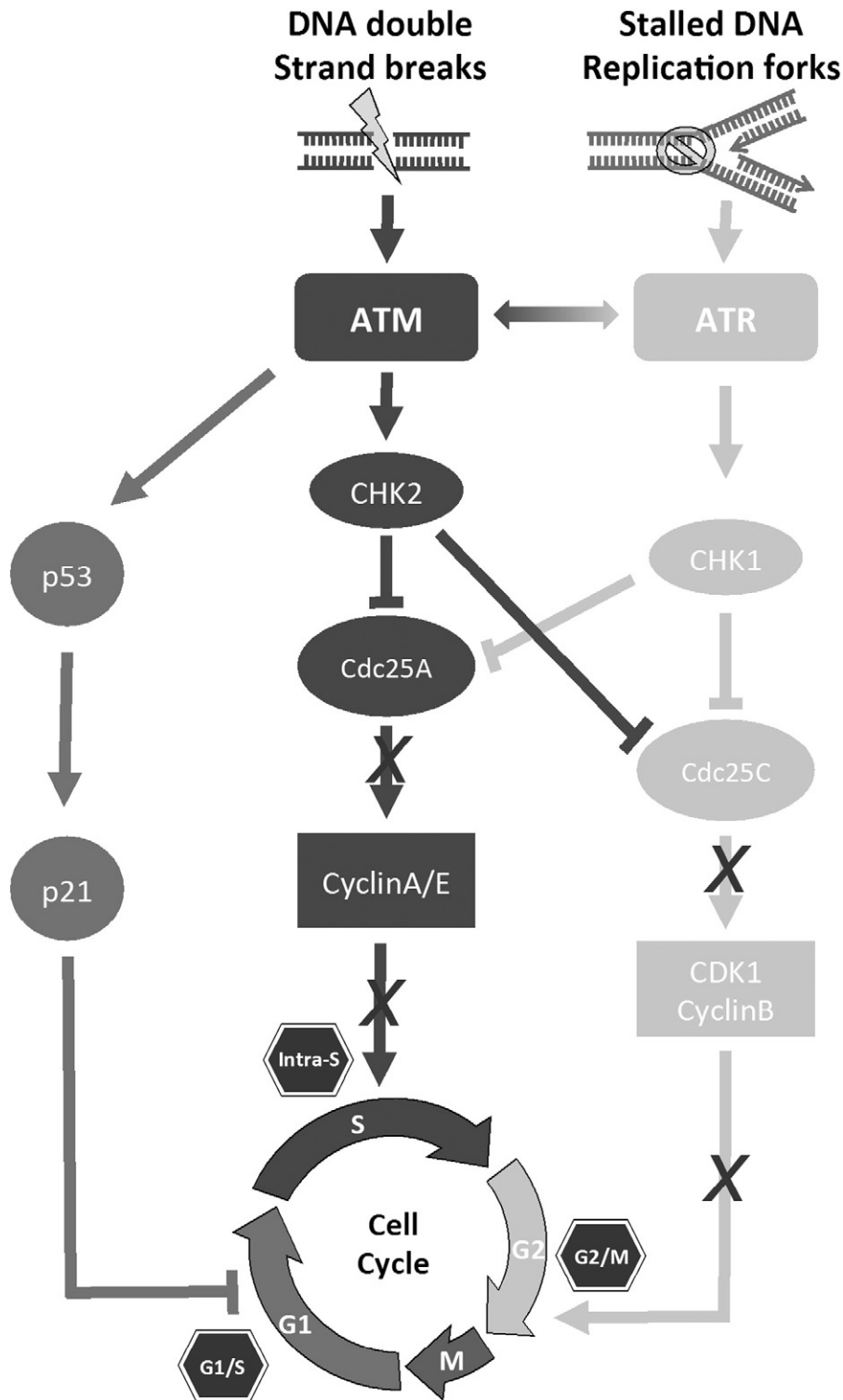

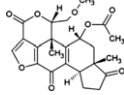
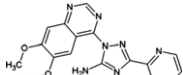
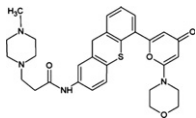


Fig. 2. Overview of ATM- and ATR-dependent DNA damage induced cell cycle checkpoint signalling. The ATM and ATR kinases are activated by the presence of DNA double strand breaks or DNA replication stress, respectively. Cell cycle progression is halted primarily through ATM and ATR dependent phosphorylation of p53, CHK1 and CHK2. G1/S cell cycle arrest is primarily mediated through a p53-dependent increase in p21, a cyclin-dependent kinase inhibitor. Key targets of CHK1/2 include the Cdc25 phosphatases that control the activity of specific cyclin-CDK complexes, which in turn regulate progression through S-phase and entry into mitosis. Phosphorylation of Cdc25 phosphatases by CHK1 or CHK2 inhibits their activity ensuring that CDK-cyclin complexes are not activated. This leads to cell cycle arrest either in S-phase or at the G2/M boundary. There are overlaps between ATM- and ATR-dependent checkpoint signalling. Notably, both CHK1 and CHK2 can phosphorylate Cdc25A and Cdc25C, depending on the source of genotoxic stress and there is direct crosstalk between ATM and ATR following their activation.

gene defective in the autosomal recessive human hereditary disorder ataxia-telangiectasia (A-T) (Savitsky et al., 1995). This disease, caused by a loss of ATM function, is characterized by progressive cerebellar degeneration, telangiectasia, immunodeficiency, genomic instability,

cancer susceptibility and profound sensitivity to ionising radiation (IR) (Taylor et al., 1975; Lavin, 2008). It is primarily this hypersensitivity of ATM-defective cells to IR that has sparked considerable interest in ATM as therapeutic target for cancer therapy (see Table 1).

Table 1
ATM inhibitors.

Compound	Targets	Description	References
Caffeine 	ATM/ATR	Caffeine sensitises cells to genotoxic modalities, particularly IR. Caffeine shows an increased radiosensitising effect in cells with p53-deficiency.	Powell et al., 1995; Yao et al., 1996; Bracey et al., 1997; Blasina et al., 1999; Sarkaria et al., 1999
Wortmannin 	ATM, PI3K, DNA-PKcs	Radiosensitises cells in vitro through inhibition of both ATM and DNA-PKcs Highly toxic in vivo	Price and Youmell, 1996; Sarkaria et al., 1998; Karve et al., 2012
CP-466722 	ATM	Highly selective ATM inhibitor which radiosensitises cells in vitro First report that transient inhibition of ATM is sufficient to confer radiosensitivity.	Rainey et al., 2008
KU-55933 	ATM	First potent and selective ATM inhibitor Confers marked sensitisation to IR and DNA DSB inducing chemotherapeutics Not suitable for in vivo use due to high lipophilicity	Hickson et al., 2004
KU-60019 	ATM	Analogue of KU-55933 with improved potency and solubility in aqueous solutions. Potently inhibits ATM downstream signalling and sensitises cells to IR in vitro Radiosensitised glioblastoma cells to IR in vivo following injection of the compound directly into the tumour. Possesses poor bioavailability in vivo otherwise	Golding et al., 2012, 2009 Biddlestone-Thorpe et al., 2013
KU-559403 	ATM	First potent and selective ATM inhibitor with good enough solubility and bioavailability to allow for in vivo studies Confers radiosensitivity in vitro Greatly increased the anti-tumour activity of etoposide and irinotecan in xenograft models	Batey et al., 2013

Early studies have demonstrated that caffeine, a methyl xanthine which inhibits the function of both ATM and ATR, sensitises cancer cells to the lethal effects of genotoxic modalities, particularly IR (Blasina et al., 1999; Sarkaria et al., 1999). Interestingly, this sensitising effect was more pronounced in cells defective in p53, indicating that ATM and/or ATR inhibition might be particularly effective for the treatment of p53-deficient tumours (Powell et al., 1995; Yao et al., 1996; Bracey et al., 1997). Despite the value of caffeine as an experimental tool for in vitro studies, it is not a clinically useful radiosensitising agent due to systemic toxicity at the doses required for radiosensitisation and the low serum levels that can be achieved in patients (Newton et al., 1981).

Wortmannin, a drug originally described as inhibitor of PI3K family members, has also been described as a potent radiosensitiser (Price & Youmell, 1996), an effect subsequently shown to be mediated through potent inhibition of both ATM and DNA-PKcs (Sarkaria et al., 1998). However, similar to caffeine, a lack of selectivity and high in vivo toxicity has hindered further development of this drug for the clinic (Karve et al., 2012).

In 2008, Rainey et al. published the results of a targeted compound library screen for potential inhibitors of the ATM kinase. In this study, the compound CP466722 was identified as a highly selective and rapidly reversible ATM inhibitor, which did not inhibit PI3K or related PIKK family members. Most importantly, the authors were able to not only validate the radiosensitising effect mediated by ATM inhibition, but also showed that transient inhibition of ATM is sufficient to achieve a significant increase in radiation-induced cytotoxicity (Rainey et al., 2008). This raises the possibility that clinically relevant radiosensitisation may not require

prolonged ATM inhibition which could help to reduce side effects in a clinical setting.

Starting out from the chemical structure of the PI3K inhibitor LY294002, KuDOS Pharmaceuticals (in 2005 acquired by AstraZeneca) developed the first potent and selective ATM inhibitor: KU-55933. In a study published in 2004, Hickson et al. demonstrated, that KU-55933 confers marked sensitisation to ionising radiation and DNA DSB-inducing chemotherapeutics, such as the topoisomerase II inhibitors etoposide and doxorubicin, in cancer cells. Importantly, in cells derived from A-T patients, which express no functional ATM, no radiosensitisation was observed, further confirming the selectivity of the compound for ATM (Hickson et al., 2004).

To improve the pharmacokinetics and bio-availability, the core structure of KU-55933 was further optimised which led to the development of KU-60019, which, like KU-55933, is an ATP-competitive ATM inhibitor. It was shown to be more effective at blocking radiation-induced phosphorylation of ATM downstream targets than KU-55933 and to possess greater potency as radiosensitiser (Golding et al., 2009). Again, A-T fibroblasts were not radiosensitised by the compound, arguing for ATM being specifically targeted. This and further in vitro studies demonstrated potent radiosensitisation of several glioblastoma cell lines by KU-60019 – a promising observation given that glioblastoma cells are usually very resistant to radiotherapy. Notably, viability of cultured human astrocytes, which are terminally differentiated and not actively dividing, was not affected by short-term exposure to KU-60019. This observation suggests that ATM inhibition alone is not toxic for normal brain tissues outside the radiation field (Golding et al., 2012). Even though KU-60019 showed better solubility in aqueous

solutions than KU-55933, bio-availability was still poor. When administered intraperitoneally or orally, systemic plasma levels only reach low micromolar concentrations (Biddlestone-Thorpe et al., 2013), limiting utility for *in vivo* studies. Biddlestone-Thorpe et al. bypassed this limitation by directly injecting KU-60019 into orthotopic gliomas grown in mice. They showed that ATM inhibition by KU-60019 markedly radiosensitised the glioma xenografts *in vivo*, leading to a significant increase in survival time of the mice. Importantly, KU-60019 showed an even greater radiosensitising effect in p53-mutant glioma, resulting in extended survival and, in some cases, apparent cure of the treated mice (Biddlestone-Thorpe et al., 2013). Though KU-60019 may not be of clinical utility, this proof-of-principle study provided evidence that pharmacological ATM inhibition can induce a potent radiosensitisation of cancer cells *in vivo*.

Recently, KU-59403, another ATM inhibitor from this class of compounds, has been described. This compound not only possesses improved potency over KU-55933, it also exhibits improved solubility and bio-availability, allowing for the study of effects of pharmacological ATM inhibition in animal models of human cancer. Batey et al. demonstrated that following administration to mice, KU-59403 showed good tissue distribution. In subcutaneous tumour xenografts, concentrations above those required for *in vitro* activity were reached and maintained for at least 4 h (Batey et al., 2013), making this the first reported *in vivo* active ATM inhibitor. In this study, it was shown that while KU-59403 alone had no impact on tumour growth rate, it greatly enhanced the anti-tumour activity of the topoisomerase inhibitors etoposide and irinotecan. Notably, the authors found the presence of KU-59403 at the time of etoposide dosing necessary to observe the chemosensitising effect. In contrast, delaying administration of KU-59403 by only 4 h completely abolished chemosensitisation. Contrary to Biddlestone-Thorpe et al., Batey and colleagues found that chemo- and radiosensitisation by ATM inhibition was not p53-dependent. Possible causes for this discrepancy include the use of different inhibitors and administration routes or the use of different tumour models (orthotopic glioma vs. subcutaneous colon cancer models). A contributing factor could also be the nature of the p53 mutations that the cancer cells carry as loss of function, dominant negative or gain of function mutations in p53 may affect therapeutic responses of cancer cells in different ways. Also, the increased sensitivity of p53-deficient glioma cells to ATM inhibition in combination with ionising radiation observed by Biddlestone-Thorpe was only seen *in vivo* and not *in vitro*. The radiosensitising effect of KU-59403, however, was only studied *in vitro*. Further studies will be required to address the question if the functional status of p53 plays a role in the radiosensitising potency of ATM inhibitors.

Despite the fact that no ATM inhibitors are in clinical development yet, the *in vitro* studies carried out to date clearly show that pharmacological ATM inhibition has great potential as a cancer therapy in combination with radiotherapy or certain chemotherapeutic drugs (like topoisomerase inhibitors). The continuous improvement and optimisation of chemical structures and screening of compound libraries have led to the development of increasingly potent and specific inhibitors that show improving properties for *in vivo* use. These developments are clearly encouraging for further development of ATM inhibitors for the clinic.

There are however, concerns of potential side-effects that should also be taken into account. Even though some studies pointed towards a potentially greater radiosensitising effect in p53-deficient tumours, ATM inhibition radiosensitises cells in general, which raises the concern of normal tissue toxicity. It should also be noted that pharmacological ATM inhibition appears to cause a different cellular phenotype than lack of ATM protein expression. Choi et al. showed that following exposure to IR, repair of damaged DNA replication forks is normal in A–T cells, which lack ATM protein, but is defective in wild-type cells when ATM is inhibited by KU-55933 or KU-60019. This effect of ATM inhibitor treatment was not observed in A–T cells (White et al., 2010).

The authors hypothesised that “kinase-inhibited ATM” presents a physical impeding to sister chromatid exchange, a mechanism of homologous recombination repair (HRR), at DSBs at damaged replication forks (Choi et al., 2010; White et al., 2010).

Interestingly, it has been shown recently that the expression of kinase dead (kd) ATM protein is more detrimental to cells than loss of ATM expression (Yamamoto et al., 2012). While ATM knockout mice have long been known to be viable, yet recapitulating many of the symptoms characteristic for A–T (Barlow et al., 1996; Elson et al., 1996), expression of physiological levels of kd ATM led to early embryonic lethality in mice. It has been suggested that this is due to the binding of catalytically inactive ATM to sites of DNA DSBs, thereby blocking those sites for proteins mediating alternative routes of DNA damage repair, and causing disturbance of the DDR and persistence of DNA damage (Yamamoto et al., 2012). ATP-competitive ATM inhibitors like KU-55933 might act in a similar way as kd ATM protein, which, upon prolonged exposure, may cause greater side effects *in vivo* than a loss of ATM protein expression would. Further studies will need to address this question, but the possibility should be considered for future clinical development of ATM inhibitors.

However, given the observations that (i) transient inhibition of ATM is sufficient to achieve radiosensitisation *in vitro* (Rainey et al., 2008) and (ii) ATM must be inhibited at the time of etoposide administration (Batey et al., 2013) to achieve chemosensitisation *in vivo*, there is strong indication that short-term treatment with optimally-scheduled ATM inhibitors might be sufficient to achieve chemo- or radiosensitisation.

In addition to the clinical use of ATM inhibitors as radiosensitisers, ATM deficiency in tumours might be exploitable as an “intrinsic radiosensitiser”. As mentioned earlier, ATM is frequently mutated in a variety of cancer types (Cancer Genome Atlas Research Network, 2012a, 2012b, 2012c, 2014). However, the large size of the ATM gene renders routine DNA sequencing a challenging diagnostic tool and a large proportion of the ATM mutations reported to date are missense variants, which occur across the entire length of the ATM protein, with no apparent hotspots. Predicting the consequences of such mutations on protein stability and activity is challenging without functional studies. Studies on ATM missense mutations found in ataxia–telangiectasia (A–T) patients have shown, however, that missense changes can lead to a reduction in ATM protein expression and that loss of ATM activity is often associated with reduced ATM protein levels (Sandoval et al., 1999; Lavin et al., 2004; Mitui et al., 2009; Jacquemin et al., 2012). In our own studies we have demonstrated that cancer-associated ATM mutations can lead to a reduction or loss of ATM protein expression and consequently impairment of the ATM signalling pathway. Furthermore we were able to show that analysis of ATM protein expression by immunohistochemistry may be a valuable clinical tool to identify a patient subgroup with low or absent ATM protein levels (Weber et al., 2014). Furthermore, studies in locally advanced breast cancer have demonstrated that the ATM gene is a potential target for epigenetic silencing (Vo et al., 2004). Hypermethylation of the ATM promoter resulting in decreased protein levels and increased radiosensitivity has been described for colorectal and glioma cell lines (Kim et al., 2002; Roy et al., 2006). A report by Tribius et al. suggested a correlation between ATM protein levels and radiosensitivity in primary glioblastoma cells in culture (Tribius et al., 2001).

Identifying loss of ATM function in tumour cells might therefore allow for the identification of a patient subset that could receive increased benefit from radiation therapy. However, this possibility will have to be addressed in future studies.

7. Ataxia–telangiectasia and Rad3 related as a therapeutic target

Several concerns revolving around functional inhibition of ATR have hindered the exploitation of ATR as therapeutic target in cancer therapy and delayed the development of specific ATR kinase inhibitors. It was anticipated that pharmacological inhibition of ATR might not be

tolerated in vivo since ATR has been shown to be an essential gene. ATR knockout in mice leads to early embryonic lethality (Brown & Baltimore, 2000; de Klein et al., 2000). In humans, mutations in ATR lead to a rare hereditary disorder, Seckel syndrome (O'Driscoll et al., 2003; Alderton et al., 2004). However, the ATR mutations causing Seckel syndrome are hypomorphic, with only a partial loss of gene function. While there are numerous human hereditary diseases which are caused by a loss of protein expression of DDR proteins like ATM (ataxia–telangiectasia) or NBS1 (Nijmegen breakage syndrome), it appears that mutations in ATR are only compatible with viability when heterozygous or hypomorphic. This further supports the concept that some ATR function is essential for the development and viability of multicellular organisms.

Furthermore, ATR activity is required in all proliferating cells during normal S-phase to ensure proper DNA replication and maintenance of genomic stability. Ruzankina et al. demonstrated that depletion of ATR in adult mice leads to defects in tissue homeostasis through acute cellular loss in tissues in which continuous cell proliferation is required for maintenance (Ruzankina et al., 2007). Furthermore, in a mouse model of Seckel syndrome, the partial loss of ATR leads to the induction of substantial replication stress, leading to accelerated ageing thereby limiting the lifespan of the mice (Murga et al., 2010). These observations may be explained by the fact, that even in the absence of replication stress-inducing agents, some replication fork stalling can occur during normal replication, for example at common fragile sites or repetitive sequences (Mirkin & Mirkin, 2007). Common fragile sites are large chromosomal regions that are thought to be particularly difficult to replicate. It has been shown that ATR is critical for fragile-site stability and that ATR-deficient cells have high levels of fragile site breakage resulting in the induction of DNA double strand breaks (Casper et al., 2002). This finding is consistent with the observation that ATR knockout leads to chromosomal fragmentation and cell death which are thought to be the underlying reason for embryonic lethality (Brown & Baltimore, 2000). The observed impediments of normal DNA replication and induction of DNA DSBs following ATR depletion raise the possibility that pharmacological ATR inhibition could cause severe side effects due to toxicity on highly proliferative normal tissues, especially if ATR inhibition was combined with drugs that cause replication stress. However, several studies have indicated that ATR inhibition might be preferentially cytotoxic for cancer cells, thereby raising the possibility of a therapeutic window for ATR inhibitors in cancer therapy. A recent study in a mouse model of Seckel syndrome demonstrated that the detrimental effects of ATR-deficiency on cell viability may be ameliorated by p53 since loss of p53 function exacerbated the accumulation of replication stress when ATR signalling was compromised (Murga et al., 2010). Functional loss of p53 was also found to profoundly aggravate the severity of ATR loss in adult mice. Simultaneous depletion of p53 and ATR exacerbated tissue degeneration, accompanied by the induction of high levels of DNA damage, and accelerated lethality of the mice (Ruzankina et al., 2009). These findings point towards an important role of p53 in the cellular response to ATR inhibition and raise the possibility that p53-deficient tumours, which comprise a high proportion of cancer cases, may show increased sensitivity to ATR inhibition compared with non-tumour tissue.

Several studies have demonstrated that depletion of functional ATR increases the sensitivity of cancer cells to oncogene-induced replication stress thereby hindering tumour growth and inducing extensive cell death (Gilad et al., 2010; Murga et al., 2011; Schoppy et al., 2012). Importantly, Schoppy et al. found that hypomorphic ATR signalling (reduced to 10% of normal levels) was sufficient to induce synthetic lethality in oncogenic RAS-driven tumours, while only minimally affecting normal bone marrow and intestinal homeostasis (Schoppy et al., 2012). This finding suggests that a low level of ATR activity may be sufficient to sustain viability of highly proliferative adult tissues and at the same time raises the possibility that complete inhibition of ATR kinase activity may not be required to cause substantial and selective toxicity in cancer cells.

Most tumour cells are defective in the DNA damage induced G1 cell-cycle checkpoint through, for example, mutations in p53 or other components of the p53 pathway. This leads to a reliance of the cells on the intra-S-phase and G2/M checkpoints to arrest the cell cycle following DNA damage induction to allow for repair of the damage and consequently cell survival (Kastan et al., 1992). Inhibition of ATR, which is an important mediator of the intra-S-phase and G2/M cell cycle arrest in such cells would therefore lead to a general loss of DNA damage checkpoints, causing DNA damage accumulation and pre-mature entry into mitosis leading to mitotic catastrophe and cell death. G1 checkpoint-proficient cells, however, would be spared.

Several proof-of-principle studies utilising expression of kinase dead ATR protein have demonstrated that functional loss of ATR leads to abrogation of the DNA damage-induced G2/M cell cycle arrest and sensitisation of cells to IR and a variety of DNA damaging chemotherapeutic agents (Cliby et al., 1998; Nghiem et al., 2002; Caporali et al., 2004). Indeed, caffeine, an inhibitor of both ATM and ATR, sensitises cells to IR and provides further support to these findings (Blasina et al., 1999; Sarkaria et al., 1999). Overall, these data encouraged the search for, and the development of, more potent and selective ATR inhibitors (Table 2).

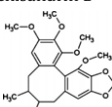
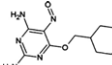
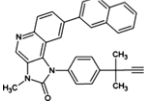
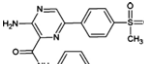
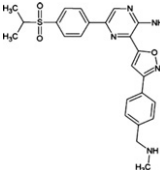
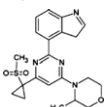
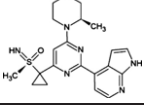
The first report on ATR-selective small-molecule inhibitors was published in 2009. Nishida et al. reported that Schisandrin B, a naturally-occurring dibenzocyclooctadiene lignan found in the medicinal herb *Schisandra chinensis* was a selective inhibitor of ATR (Nishida et al., 2009). The authors demonstrated that Schisandrin B was able to abrogate UV-induced intra-S-phase and G2/M cell cycle checkpoints and increase the cytotoxicity of UV radiation in human lung cancer cells. However, the inhibitory potency against ATR was weak and required the use of high drug concentrations (30 μ M for cellular assays).

A more potent ATR inhibitor, NU6027, was reported in 2011 and was demonstrated to sensitise several breast and ovarian cancer cell lines to IR and several chemotherapeutic agents (Peasland et al., 2011). But, this compound was originally developed as a CDK2 inhibitor and is not selective for ATR.

Also in 2011, Toledo et al. reported the results of a cell-based compound library screening approach for the identification of potent ATR inhibitors (Toledo et al., 2011). One of the compounds identified to possess significant inhibitory activity against ATR kinase was NVP-BE2235, a drug originally introduced as a highly potent dual inhibitor of PI3K and MTOR with considerable in vivo anti-tumour activity (Maira et al., 2008). NVP-BE2235 has been demonstrated to markedly radiosensitive Ras-overexpressing tumours (Konstantinidou et al., 2010). However, in light of the finding that it also inhibits ATR (and to a lesser extent ATM and DNA-PKcs), it seems likely that inhibition of the DDR kinases rather than PI3K or MTOR contributed to the observed effects. The aforementioned study by Gilad et al. which demonstrated that ATR-depletion is particularly cytotoxicity in cells that overexpress oncogenic Ras is in agreement with this notion (Gilad et al., 2010). ETP-46464 and Torin 2 are additional examples of compounds which possess potent ATR inhibitory activity, but lack selectivity (Liu et al., 2011; Toledo et al., 2011).

A high-throughput screening campaign led to the discovery of the first series of both potent and selective ATR kinase inhibitors by Vertex Pharmaceuticals (Charrier et al., 2011). One of these compounds, VE-821, was shown to be a potent ATP-competitive inhibitor of ATR with minimal cross-reactivity against the related PIKKs ATM, DNA-PKcs and MTOR (Reaper et al., 2011). VE-821 inhibited phosphorylation of the ATR downstream target CHK1 at Ser345 and showed strong synergy with genotoxic agents from multiple classes in the colorectal cancer cell line HCT116. The observed chemosensitisation was most pronounced with DNA cross-linking agents such as cisplatin, and was further enhanced by knockdown of p53 expression, in ATM-deficient cells or in combination with the specific ATM inhibitor KU-55933. Importantly, VE-821 cytotoxicity in normal cells appeared minimal, causing only a reversible growth arrest without significant induction of cell death (Reaper et al., 2011). These findings

Table 2
ATR inhibitors.

Compound	Targets	Description	References
Schisandrin B 	ATR	Selective, though weak ATR inhibitor. Abrogates the UV-induced S and G2/M cell cycle checkpoints and increases sensitivity of cancer cells to UV radiation	Nishida et al., 2009
NU6027 	CDK2, ATR	NU6027 was originally developed as CDK2 inhibitor, but also potently inhibits ATR activity. Has been demonstrated to chemo- and radiosensitise several breast and ovarian cancer cell lines	Peasland et al., 2011
NVP-BE2253 	PI3K, MTOR, ATR, ATM, DNA-PKcs	Originally developed as dual PI3K/MTOR inhibitor, also possesses substantial activity against ATM, ATR, and DNA-PKcs. Has been demonstrated to potently radiosensitise RAS-overexpressing tumours	Maira et al., 2008; Toledo et al., 2011 Konstantinidou et al., 2010
VE-821 	ATR	First potent and selective ATR inhibitor. Chemosensitises cancer cells to various chemotherapeutics in vitro. Chemosensitisation most potent for cross-linking agents. Potent in vitro radiosensitiser. Shows increased single-agent toxicity in hypoxic cells	Charrier et al., 2011; Reaper et al., 2011; Pires et al., 2012; Prevo et al., 2012; Huntoon et al., 2013
VE-822 (VX-970) 	ATR	Analogue of VE-821 with increased potency and improved bioavailability. First ATR inhibitor to enter clinical development. Increased anti-tumour activity with IR and gemcitabine in xenograft models of pancreatic cancer. Chemosensitises lung cancer cells to several chemotherapeutic drugs in vitro. Increases the anti-tumour activity of cisplatin in vivo	Fokas et al., 2012; Hall et al., 2014 ClinicalTrials.gov: NCT02157792
AZ20 	ATR	Potent and selective ATR inhibitor. Possesses substantial single agent activity against MRE11-deficient cancer cells in vivo at well tolerated doses	Jacq et al., 2012; Foote et al., 2013
AZD6738 	ATR	Analogue of AZ20 with improved solubility, pharmacodynamics and bioavailability. Significantly increases anti-tumour activity of IR or carboplatin in vivo. Shows single-agent activity in ATM-deficient xenograft models. Currently in Phase I clinical development	Guichard et al., 2013; Jones et al., 2013 ClinicalTrials.gov: NCT02223923

are further supported by a study published earlier the same year, which demonstrated that genetic inhibition of ATR expression selectively enhanced cisplatin sensitivity in human colorectal cancer cells with inactivated p53 (Sangster-Guity et al., 2011).

Together these studies further promote the concept that G1 checkpoint-deficient cancer cells might be more sensitive ATR kinase inhibition, especially in combination with genotoxic treatments. VE-821 has since been used in several studies and has consistently been shown to sensitise a variety of cancer cell lines to IR and chemotherapy (Pires et al., 2012; Prevo et al., 2012; Huntoon et al., 2013). Pires et al. demonstrated, that VE-821 enhanced IR-induced cytotoxicity in a panel of 12 human cancer cell lines. Notably, VE-821 radiosensitised cancer cells under severe hypoxia and over a range of oxygen concentrations (Pires et al., 2012). This is of particular interest, as hypoxic tumour cells are more resistant to radiotherapy (Wouters & Brown, 1997; Sprong et al., 2006) and therefore represent a major obstacle for the efficacy of radiotherapy. However, whether such a strategy is also effective in vivo is yet to be demonstrated.

VE-822, an analogue of VE-821 with increased potency and selectivity against ATR, increased solubility and good pharmacokinetic properties was shown to potently radiosensitise pancreatic cancer cell lines in vitro. Furthermore, VE-822 treatment profoundly radiosensitised xenograft models of human pancreatic cancer and further increased the growth delay induced by IR combined with gemcitabine. Importantly, VE-822 was well tolerated in mice and did not enhance toxicity in normal cells and tissues (Fokas et al., 2012). VE-822 was the first selective ATR inhibitor to enter clinical development, and is now known as VX-970.

In a recent study, published by Vertex Pharmaceuticals, VX-970 (VE-822) was shown to markedly sensitise a panel of non-small cell lung cancer cell lines, but not normal cells, to multiple DNA damaging drugs, namely cisplatin, oxaliplatin, gemcitabine, etoposide and SN38, the active metabolite of irinotecan. The sensitising effect of VX-970 was most evident with cisplatin and gemcitabine co-treatment, where >75% of the 35 tested cell lines were sensitised. Consistent with previous reports, the observed chemosensitisation was more pronounced in cells with p53-deficiency than in cells with retained p53 activity. In patient-derived lung tumour xenograft models, VX-970 significantly improved responses to cisplatin (in six out of the seven models) (Hall et al., 2014). These data suggest that VX-970 may have the potential to increase the efficacy of DNA damaging therapy in patients with lung cancer. A phase I clinical trial to assess the safety, tolerability and pharmacokinetics of VX-970 in combination with cytotoxic chemotherapy is currently ongoing (ClinicalTrials.gov: NCT02157792).

A second ATR inhibitor currently in clinical development is AZD6738, developed by AstraZeneca. AZD6738 is an analogue of AZ20, a potent and selective ATR inhibitor which has been shown to possess substantial in vivo single agent activity in MRE11A-deficient LoVo xenografts at well tolerated doses (Jacq et al., 2012; Foote et al., 2013). AZD6738 possesses significantly improved solubility, bioavailability and pharmacokinetic properties compared to AZ20 and is suitable for oral dosing (Jones et al., 2013). It inhibits phosphorylation of the ATR downstream target CHK1 while increasing phosphorylation of the DNA DSB marker γ H2AX in vitro. In vivo combination studies with carboplatin or IR demonstrated significantly increased anti-tumour growth inhibitory activity with this

compound. Furthermore, AZD6738 showed single-agent anti-tumour activity in ATM-deficient but not ATM-proficient xenograft models (Guichard et al., 2013; Jones et al., 2013). This anti-tumour activity was associated with a persistent increase in γ H2AX staining in tumour tissue but only a transient increase in normal tissues such as bone marrow or gut. This suggests that a favourable therapeutic index could be achieved, which is encouraging for the further development of this compound for the clinic. A phase I clinical trial to assess the safety of AZD6738 alone and in combination with radiotherapy in patients with solid tumours is currently recruiting (ClinicalTrials.gov: NCT02223923).

8. Synthetic lethal approaches

In addition to the potential utility of ATM and ATR as chemo- or radiosensitisers, recent studies suggest that such compounds may have single agent activity in certain subsets of patients through induction of synthetic lethality. Two genes are considered synthetically lethal, if mutation or inactivation of either gene or gene product alone has no effect on cellular viability, whereas simultaneous defects in both genes/gene products lead to cell death (Kaelin, 2005).

The observed single-agent anti-tumour activity of AZD6738 in ATM-deficient but not ATM-proficient xenograft models suggests that such a synthetic lethal interaction could exist between the ATM and ATR signalling pathways (Guichard et al., 2013; Jones et al., 2013), potentially due to the overlapping and cooperating roles of these pathways in coordinating cell cycle progression with DNA repair. In our own studies we were able to show that non-small cell lung cancer cells deficient in both ATM and p53 are particularly sensitive to ATR inhibition *in vitro*, suggesting that the functional status of p53 may be important in this setting as well (Weber et al., 2013). Similarly, PARP inhibitors possess single agent activity in ATM-deficient tumour cells *in vitro* and *in vivo* (Aguilar-Quesada et al., 2007; Weston et al., 2010; Williamson et al., 2010). However, the cytotoxicity of PARP inhibitors was shown to be enhanced when the function of both ATM and p53 was lost (Williamson et al., 2012; Kubota et al., 2014).

Several putative synthetic lethal interactions between the ATM or ATR signalling pathways and other DDR components have been reported. Inhibition or knockdown of ATM has been shown to be synthetically lethal in cells with defects in the Fanconi anaemia pathway, components of which are commonly mutated or lost in cancer (Kennedy & D'Andrea, 2006; Kennedy et al., 2007). ATM inhibition or -deficiency has also been shown to be synthetically lethal in combination with APE1 inhibitors (Sultana et al., 2012) or functional loss of XRCC1 (Sultana et al., 2013a), which is frequently deregulated in breast and ovarian cancers (Abdel-Fatah et al., 2013; Sultana et al., 2013a). ATR inhibition has been shown to be synthetically lethal with XRCC1 or ERCC1 loss (Sultana et al., 2013a; Mohni et al., 2014). Furthermore, the observed increased cytotoxicity of the ATR inhibitors AZ20 and AZD6738 in MRE11A mutated LoVo cells *in vitro* and *in vivo* (Jacq et al., 2012; Foote et al., 2013; Guichard et al., 2013) points towards a potential synthetic lethal interaction between ATR inhibition and functional loss of MRE11. This synthetic lethality may include other components of the MRN complex as well (Al-Ahmadie et al., 2014). Hence, pharmaceutical inhibition of ATM and/or ATR may provide the basis for the selective treatment of DDR pathway-deficient cancers.

9. Biomarkers and patient selection

To increase the success rate for drug development it has been proposed that biomarkers to identify patient subgroups likely to get the greatest benefit from new drugs should be identified early, alongside biomarkers of target inhibition (pharmacodynamic; PD), pathway modulation, and anticipated biological effect(s) (Yap et al., 2010).

Although patient selection biomarkers for ATM and ATR inhibitors have not yet been established, published *in vitro* and *in vivo* data suggest a number of potential strategies (Table 3). Increased tumour

DDR capacity has been associated with resistance to radio- and chemotherapies, for example by upregulating DNA repair capacity in response to platinum-based therapy (Martin et al., 2008; Oliver et al., 2010). Although resistance to therapy is likely to be multifactorial and highly dependent on the particular therapy under consideration (e.g. cisplatin, taxanes, radiation), conceptually, a test for increased tumour DDR capacity might identify a group of patients who would gain particular benefit from combination therapy with either an ATM or ATR inhibitor. Interestingly, elevated levels of phospho-ATM (Ser1981) prior to radiotherapy has been associated with radioresistance and poor prognosis in cervical cancer (Roossink et al., 2012). This suggests that levels of ATM autophosphorylation in tumour tissues could serve as a potential biomarker to identify patients that might get the greatest benefit from a combination of ATM inhibitor treatment and radiotherapy. As outlined above, many human tumours acquire defects in the DDR in order to tolerate DNA replication stress and genomic instability that are characteristic of oncogene activation during cancer development. These DDR defects offer the potential to use ATM or ATR inhibition as a synthetic lethal approach, in an analogous manner to the use of PARP inhibitors in BRCA1/2 defective tumours. Functional defects in several components of the DDR pathway have been reported to confer sensitivity to ATM and/or ATR inhibition, including p53 (Reaper et al., 2011), XRCC1 (Sultana et al., 2013a, 2013b), FANCD2 (Kennedy et al., 2007), MRE11A (Jacq et al., 2012; Foote et al., 2013; Guichard et al., 2013), RAD50 (Al-Ahmadie et al., 2014), BRCA1 (Albarakati et al., 2014) and ATM (Reaper et al., 2011). Whole exome/genome sequencing offers a potential method to identify patient subgroups or particular cancer types with DDR defects (Kandoth et al., 2013), although the relationships between reported mutations and loss of protein function for many of the DDR proteins have yet to be determined. Of the DDR genes, p53 is the most widely studied with an extensive literature on the functional consequences of a wide range of mutations (Petitjean et al., 2007). Therefore, p53 mutation status may provide an enrichment strategy for selecting patients for treatment with ATM or ATR inhibitors, but p53 status remains a complex biomarker to interpret (Olivier et al., 2010).

It has also been reported that defects in ATM or ATR signalling are synthetically lethal with PARP inhibition (Turner et al., 2008; Peasland

Table 3
Potential biomarkers in the development of ATM and ATR inhibitors.

Patient selection	Target inhibition	Pathway modulation
Over-expression of components of DDR pathway ^a	- pATM ^b - pKAP1 ^b	- Dysfunctional cell cycle arrest ^k - Unrepaired DNA DSBs ^l
DDR defects (intrinsic)	- pSMC1 ⁱ - ATM ^b - p53 ^b - FANCD2 ^c - XRCC1 ^d - MRE11A ^e	- Increased - DNA replication stress ^m - Premature mitosis and mitotic catastrophe ⁿ
DDR defects (conditional)	- Hypoxia ^f	
Replication stress	- Activating oncogenes (e.g. Ras, Myc) ^g	

^a Bao et al. (2006).

^b Reaper et al. (2011).

^c Kennedy et al. (2007).

^d Sultana et al. (2013a, 2013b).

^e Foote et al. (2013).

^f Pires et al. (2012).

^g Gilad et al. (2010), Schoppy et al. (2012), Murga et al. (2011).

^h Guo et al. (2014).

ⁱ Kitagawa et al. (2004).

^j Burma et al. (2001).

^k Cliby et al. (1998).

^l Kühne et al. (2004).

^m Murga et al. (2011).

ⁿ Blasina et al. (1999).

et al., 2011; Yap et al., 2011; Michels et al., 2014), suggesting that combined inhibition of PARP and ATM or ATR may be an effective therapeutic strategy. In addition to intrinsic defects in DDR, tumour cells may also acquire conditional DDR defects. Under hypoxic conditions, the expression of several proteins involved in homologous recombination is down-regulated, conferring sensitivity to ATR inhibition (Chan et al., 2010; Pires et al., 2012). Potentially, patient subgroups with high levels of tumour hypoxia could be identified by immunohistochemistry (Loncaster et al., 2001), using gene expression arrays (Eustace et al., 2013), or by non-invasive imaging (e.g. with 18F-Misonidazole PET (Eschmann et al., 2005)).

In the early stages of clinical development, it is crucial to demonstrate that a new drug inhibits its target sufficiently to modulate relevant pathways to elicit a biological response. For ATM inhibitors, a number of potential measures of target inhibition have been identified, including ATM autophosphorylation, or phosphorylation of the ATM downstream targets p53, KAP1, SMC1, CHK2, and H2AX (γ H2AX) (Shiloh & Ziv, 2013; Guo et al., 2014). A caveat is that several of these markers, including p53 and γ H2AX are not specific measures of ATM kinase activity as they may also be targets of other kinases, including DNA-PKcs and ATR (Hammond et al., 2003; Mukherjee et al., 2006). The use of a panel of these markers is likely to be most informative in evaluating the activity of ATM inhibitors in the clinic (Bartkova et al., 2005; Kozlov et al., 2011).

CHK1 phosphorylation is the most widely used preclinical biomarker of ATR kinase activity (Fokas et al., 2012; Pires et al., 2012; Hall et al., 2014). However, ATM can also phosphorylate CHK1 (Gatei et al., 2003; Sørensen et al., 2003; Helt et al., 2005), albeit to a lesser extent and a recent study in ovarian cancer has suggested that CHK1 phosphorylation status may not offer a reliable marker for inhibition of the ATR-CHK1 pathway (Huntoon et al., 2013). Other, less direct, measures of ATR inhibitor activity could include increased DNA replication stress markers such as pan-nuclear γ H2AX (Jacq et al., 2012; Foote et al., 2013; Guichard et al., 2013; Jones et al., 2013) or nuclear foci staining of RPA or RAD51, a marker for homologous recombination repair, but these are unlikely to be specific pathway markers of ATR inhibition.

Finally, the biological consequence of ATM/ATR target and pathway inhibition, either in combination with genotoxic therapy, or as a synthetic lethal monotherapy in DDR-defective tumours is selective tumour cell death which would be expected to produce tumour shrinkage in the clinical setting measurable by standard RECIST criteria (Fojo & Noonan, 2012) with the potential for assessing early circulating markers of apoptosis (Ward et al., 2008).

10. Conclusion

A broad range of preclinical research and an extensive literature of underpinning science support the development of ATM and ATR kinase inhibitors in two distinct clinical settings – as a monotherapy aiming for synthetic lethal responses in tumours with intrinsic or conditional DDR defects, and in combination with chemo- or radiotherapy. Each setting has distinct challenges. Identifying the right patients will be critical if the synthetic lethal strategy is to be successful. Current sequencing techniques can identify mutations in DDR genes that may impact on the response of cancer cells to ATM or ATR inhibitors. However, without functional studies, the consequences of these mutations are difficult to predict for proteins such as, for example, ATM, MRE11, RAD50 or FANCD. In addition, DDR defects may arise by epigenetic and/or post-transcriptional mechanisms. Developing biomarkers that can robustly determine the functional status of DDR pathways in tumours and help differentiate between deleterious and benign mutations will therefore be essential.

For combination studies, a key issue is likely to be concern over the potential for increased normal tissue toxicity, and identifying the combinations and schedules that have the greatest potential for tumour selective effects is a priority area of research that needs further investigation. Selective ATR inhibitors are currently in Phase 1 clinical development

and it seems likely that ATM inhibitors with good pharmacological properties will become available in the near future. Notably, inhibitors of other DDR targets including PARP, CHK1/2, WEE1, and DNA-PKcs are also in clinical development and it will be of great interest to follow the progress of these approaches and to use the emerging results to better guide the future clinical development of ATM and ATR inhibitors.

Conflict of interest

The authors declare that there are no conflicts of interest.

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