Chapter 8
Targeting ATM for Cancer Therapy: Prospects for Drugging ATM

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Abstract As discussed in the previous chapter, the rationale for inhibition of ATM as a therapeutic strategy in cancer is both scientifically sound and well explored. The use of experimental models and, thereafter, the availability of tool compounds to inhibit the target, has allowed the role of ATM in cell signalling to be refined and has highlighted the potential utility of ATM inhibition for therapeutic intervention. The role of ATM as the central DNA damage response (DDR) protein, the high sensitivity of cells from A-T patients, who lack functional ATM, to IR and DNA damaging chemotherapy, and the consequences of knocking down ATM in otherwise proficient cells, have been well described and support ATM as a pharmaceutical target of interest. The somewhat atypical nature of ATM (a member of the PIKK family of kinases), combined with the size of the protein, have brought some unique challenges and opportunities to the discovery of inhibitors of ATM. The development of robust, high-throughput biochemical assays for ATM inhibition has proved challenging, thereby requiring the establishment of less conventional assays to facilitate drug discovery efforts. However, the availability of early compounds that were shown to share features of ATM loss (i.e. bringing about sensitisation of cells to IR induced cell damage and death), helped advance the process and over the past decade the research into ATM inhibition has advanced as the quality of available inhibitors has improved. In this chapter, we will explore the evolution of ATM inhibitors from crude but effective tools, through highly selective tool compounds and ultimately to the development of compounds with potential clinical utility as therapeutics for cancer patients.

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8.1 Introduction

The biology, rationale and scope of ATM inhibitors has been very well detailed in the previous chapter but it is worth highlighting the key points of the role of ATM in the DNA Damage Response (DDR), as these features relate to the identification of inhibitors of ATM cellular activity. Ataxia telangiectasia (A-T) is a rare autosomal recessive disease, resulting in a syndrome of neurodegenerative disease, causing severe disability. Aside from the associated developmental and immunological effects of germline mutations of the Ataxia-telangiectasia mutated (ATM) gene, A-T patients suffer from genomic instability and cancer susceptibility, which has been linked to profound sensitivity of A-T cells to ionising radiation (IR), and radiomimetic (DNA-damaging) drugs (Lavin 2008; Lavin and Shiloh 1997; Rotman and Shiloh 1998; Taylor et al. 1975; Köcher et al. 2012, 2013). The cell cycle checkpoint defects of A–T cells result in failure to arrest cells at the G1/S boundary following exposure to ionising radiation, radiation-resistant DNA synthesis (Houldsworth and Lavin 1980; Kastan et al. 1992; Painter and Young 1980) and failure to delay mitotic entry (Beamish and Lavin 1994). Thus, inhibitors of ATM administered to ATM proficient cell lines would be expected to result in marked sensitisation to radiation induced DNA damage, disruption of cell cycle and, when combined with either radiation or chemotherapeutics that damage DNA, result in cell death (Weber and Ryan, 2015). It is important to note that the screening, characterisation and development of ATM inhibitors, as described in this chapter, is designed around and dependent upon the observed biology of the cells of A-T patients.

8.2 ATM as a Target

ATM is large protein (350 kDa) comprising 3056 residues and containing a kinase domain with a relatively high degree of similarity to the lipid kinase PI3K, hence its designation as a phosphoinositide 3-kinase (PI3K)-related kinase (PIKK) (Shiloh 2003). These atypical serine/threonine kinases also comprise ATR, DNA-PKcs, mTOR, SMG1 (and non-enzymatic TRRAP) (Fruman et al. 1998). To facilitate drug discovery efforts in identifying ATM inhibitors, assays of biological activity are required. Such assays rely on features of the biological function of ATM. Specifically, the inactive dimeric form of ATM is recruited to the site of a DSB by the DNA-end tethering MRE11-RAD50-NBS1 (MRN) complex that results in the autophosphorylation of ATM on Serine 1981 (Bakkenist and Kastan 2003; Lee and Paull 2005). This auto-phosphorylation event leads to dimer

dissociation, activation of ATM and subsequent phosphorylation of nearby histone variant H2AX on Serine 139 (γH2AX), phosphorylation of p53 on serine 15, and of Mdm2 and CHK2 (Banin et al. 1998; Marine and Lozano 2010; Matsuoka et al. 1998). Phosphorylation of p53 results in activation and accumulation in the nucleus, whereupon p53 acts as transcription factor to drive the expression of genes involved in G1/S cell cycle checkpoint activation and apoptosis (Sullivan et al. 2012). Although there are over 700 substrates phosphorylated in an ATM-dependent manner (Bennetzen et al. 2010), these key events have been used to build screening assays for ATM (Guo et al. 2014), that have ultimately led to the discovery of clinical candidate molecules described at the end of this chapter. The importance of the understanding of the biology of ATM in obtaining tractable chemical matter against the target cannot be underestimated. Whilst loss of ATM protein and ATM inhibition are not a phenocopy per se (Choi et al. 2010; Fedier et al. 2003; Foray et al. 1997; Kühne et al. 2004; Yamamoto et al. 2012), small molecule inhibitors of ATM have been shown to largely match A-T signalling events, indicating a specificity and relevant biological function to the molecules developed.

8.3 Early Inhibitors of ATM

During the earliest efforts to find chemical inhibitors of ATM, the fungal metabolite, Wortmannin (1), was identified as an inhibitor of ATM enzyme (IC_{50} = 0.15 μM) (Powis et al. 1994). Although the ATM-directed p53 response has been shown to be diminished in these experiments, optimal radiosensitisation and induction of S and G2 cell cycle phase abnormalities occurs at concentrations above those required to inhibit ATM. It is most likely that the increased sensitivity to ionizing radiation observed was manifested through the ability of Wortmannin to inhibit not only ATM but a number of PIKKs, including ATR and DNA-PK, as well as PI3K (DNA-PK IC_{50} = 0.016 μM; ATR IC_{50} = 1.8 μM; PI3K IC_{50} = 0.003 μM). (Izzard et al. 1999; Sarkaria et al. 1998; Ui et al. 1995). This chemical inhibitor approach, however, demonstrated the feasibility of achieving sensitisation to ionising radiation although did not constitute proof that this effect was driven by ATM inhibition alone. Acting as a non-competitive irreversible inhibitor, Wortmannin probably hits too many other targets to be a viable clinical agent and indeed has been demonstrated to have toxicity in in vivo experiments. Attempts to reformulate the compound for nanoparticle delivery and thereby provide some tumour targeting were unable to sufficiently modulate therapeutic index to support clinical use (Karve et al. 2012).

Caffeine (2), is a methyl xanthine that has been shown to sensitise cells to the lethal effects of genotoxic modalities, including IR (Blasina et al. 1999). The molecule is a relatively weak and non-specific ATM inhibitor (IC_{50} = 200 μM against enzyme), with similarly weak activity against a number of PIKK enzymes (ATR IC_{50} = 1100 μM, DNA-PK IC_{50} = 10,000 μM) (Sarkaria et al. 1999). In the case of caffeine and its potential use in vivo, the sensitisation occurs at an effective concentration that is clinically prohibitive; serum concentrations of 1 mM, which are

required to achieve radiosensitisation, are associated with fatal tachyarrhythmias (Sarkaria and Eshleman 2001).

The first synthetic specific inhibitor of the PIKKs was the flavonoid quercetin analogue LY294002 (3), developed by Eli Lilly (Vlahos et al. 1994). LY294002 is an ATP competitive inhibitor with modest potency against a variety of PI3K and PIKK enzymes (PI3Kα, β, γ, δ IC_{50} = 0.55, 16, 12 and 1.6 μM respectively; mTOR IC_{50} = 2.5 μM; DNA-PK IC_{50} = 2.5 μM) and has facilitated the improved understanding of the function of PI3K and PIKKs as well as providing a start-point for the subsequent development of more selective PIKK inhibitors. Although the activity of LY294002 against ATM is somewhat limited (IC_{50} > 100 μM) (Knight et al. 2004), the understanding of the binding mode of the inhibitor in PI3Kγ, in particular the importance of the interactions of the morpholine ring (Andrs et al. 2015), was instrumental in the discovery of future potent and selective tools to probe ATM biology. For this reason, LY294002 is rightly placed in a review of ATM chemical matter.

More recently the dual PI3K/mTOR inhibitor NVP-BEZ235 (4), developed by Novartis, has been shown to be a potent inhibitor of ATM. This compound was taken into clinical studies but development ultimately halted due to toxicity and poor efficacy. Although originally described as a dual mTOR/PI3K inhibitor (mTOR IC_{50} = 0.021 μM; PI3Kα, β, γ, δ IC_{50} = 0.004, 0.075, 0.005 and 0.007 μM respectively) (Maira et al. 2008), it has been shown to inhibit ATM and DNA-PK (ATM IC_{50} = 0.007 μM; DNA-PK IC_{50} = 0.005 μM). The subsequent observation of ATR inhibition (IC_{50} = 21 nM, Toledo et al. 2011), and radio-sensitisation of cells overexpressing RAS to the inhibitory effects of NVP-BEZ235 (Konstantinidou et al. 2009), have further highlighted the potential for toxicity as a result of polypharmacology and underscores the requirement for highly selective ATM inhibitors to develop as potential clinical agents.

CGK733 was originally reported as a dual ATM and ATR inhibitor, capable of reversing cellular senescence (Won et al. 2006), and has been used in a number of publications to explore ATM and ATR activity. The data supporting the original publication were called into question along with the validity of the experiments and the original publication was retracted (Won et al. 2008). Subsequent analysis of the ATM inhibitory potential of CGK733 confirmed that the compound was not an ATM inhibitor (Choi et al. 2011), and as such any data generated with this molecule should be treated with caution (Fig. 8.1).

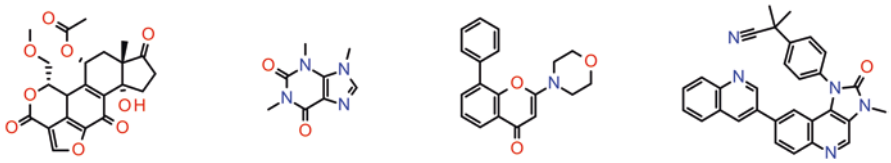


Fig. 8.1 Published structures of non-selective ATM inhibitors

8.4 Selective ATM Inhibitors as Probe Molecules

The identification of the pan PI3K/PIKK inhibitors described above has helped advance our understanding of these important targets, but the need to develop selective ATM inhibitors continued.

The compounds used in the above early studies (Caffeine and Wortmannin) are non-specific and also inhibit other phosphatidylinositol 3-kinase-related kinase (PIKK) family members, including ATR and DNA-PKcs (Sarkaria et al. 1998, 1999). Therefore, it is uncertain how much of the observed effects were due to inhibition of ATM activity. The development of more potent and selective ATM inhibitors allowed for the validation of the radio-sensitising effect mediated by pharmacological ATM inhibition in vitro.

As mentioned previously, in addition to increasing the understanding of ATM biology, these non-selective ATM inhibitors also provided important start-points for the development of selective agents. In particular, the morpholine containing scaffold of LY294002, resulted in the development of a number of PIKK targeted inhibitors, a particularly potent and selective example being that of KU-55933 (5). Through screening of a small library of molecules designed around LY294002, the chemistry teams of KuDOS Pharmaceuticals and the Northern Institute for Cancer Research (NICR), described the discovery, synthesis and characterisation of 2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one, KU-55933 (Hickson et al. 2004; Hollick et al. 2007). KU-55933 potently inhibits ATM in biochemical assays (IC_{50} = 12.9 nM). Counter-screens of KU-55933 against other members of the PIKK family demonstrated at least a 100-fold differential in selectivity (DNA-PK (IC_{50} = 2.5 μM), ATR (IC_{50} > 100 μM), mTOR (IC_{50} = 9.3 μM) and PI3K (IC_{50} = 16.6 μM)). Furthermore, in screening a commercially available panel of 60 kinases at a single concentration of 10 μM, KU-55933 did not significantly inhibit any kinase tested. The evolution of KU-55933 from LY294002 also validates the ability of small molecule ATP-competitive kinase inhibitors to display high levels of selectivity between PIKK family members, a finding further validated by the identification of potent and selective inhibitors of DNA-PK (Hollick et al. 2007).

The role of a hydrogen bond mediated interaction between the morpholine oxygen of LY294002 and the hinge region of PI3K p110γ was highlighted in a crystal structure (Walker et al. 2000). The importance of this moiety for the activity of KU-55933 against ATM was confirmed by the use of the related molecule, 2-piperidin-1-yl-6-thianthren-1-yl-pyran-4-one, KU-58050 (structure not shown), in which the morpholine unit has been replaced by a piperidine unit, thereby removing the possibility of an analogous hydrogen bonding interaction. KU-58050 was found to have significantly reduced ATM activity (IC_{50} = 2.96 μM), indicating the compound to be more than 200 times less effective as an ATM inhibitor when compared to KU-55933. In addition to confirming the importance of the morpholine oxygen for ATM inhibition in this scaffold, KU-58050 also serves as a useful negative control for ATM activity in experiments utilising KU-55933 due to the closely related molecular structure. On the basis of the structural similarity between KU-55933

(compound 5) and LY294002 (compound 3), (Izzard et al. 1999; Vlahos et al. 1994), it was assumed that the inhibition of ATM by KU-55933 would be ATP competitive. Hickson et al. (2004), described the derivation of competitive ATP binding data that was subsequently corroborated by Kevan Shokat's group, who further went on to highlight the extremely selective inhibition of ATM by KU-55933 in an analysis of a broad range of ATP competitive compounds targeting PIKK proteins (Knight et al. 2006). In this publication, the use of the homology models of a number of PIKK proteins and associated inhibitors, in particular for PI3K and ATM binding, supports the earlier model established for KU-55933 binding in the ATM pocket.

KU-55933 was the first potent and selective inhibitor of ATM to be reported and has been adopted by the research community as an effective tool for assessing the cellular role of ATM inhibition. Its use has been broadly reported in the literature in experiments to determine ATM function, basic biology and also the potential therapeutic utility of an ATM inhibitor. Consistent with the previously described biology of ATM loss resulting in sensitivity to genotoxic damage (Blasina et al. 1999; Fedier et al. 2003; Foray et al. 1997; Price and Youmell 1996; Sarkaria et al. 1998, 1999), KU-55933 was shown to sensitize HeLa cells to the cytotoxic effects of topoisomerase I and II inhibitors and to IR. The differentiation of ATM biology is helped by the selective inhibitors that, in the case of KU-55933 for example, are not further sensitising to cells that lack ATM expression (Hickson et al. 2004); data with less selective inhibitors is confounded by activity beyond ATM.

As noted in the previous chapter, the known biology of ATM loss was critical to the development of appropriate tools and ultimately, of course, to the clinical development of an ATM inhibitor. It has been shown that kinase-dead ATM acts in a dominant negative like manner such that expression of physiologically equivalent levels of kinase-dead ATM are lethal in early embryogenesis whereas A-T mice, lacking ATM, are viable (Daniel et al. 2012; Yamamoto et al. 2012). The defect in repair of damaged replication forks observed in KU-55933 treated cells (White et al. 2010), indicated that inhibition of ATM may act in a similar way to kinase-dead ATM protein (Yamamoto et al. 2012) as opposed to that observed for loss of ATM (i.e. kinase inhibited ATM physically blocks homologous recombination repair of DSBs at damaged replication forks) (Choi et al. 2010; White et al. 2010). Parallels could be drawn with the inhibition of PARP, and so-called PARP trapping, that prevents the processing of a lesion in DNA, as distinct from an absence of PARP at the site of damage (Murai et al. 2012).

This observation raises important questions when exploring the biological consequences of ATM inhibition. If 'trapping' of the repair process occurs with ATM inhibitors, could this be more detrimental in vivo than a loss of ATM protein due to the prolonged lesion? It may, therefore, be important to assess if it is possible to achieve exposures that enable sensitisation to chemo- or radiotherapy but that will not cause sustained trapping of ATM on the DNA, which may lead to adverse impacts on normal tissues. The kinetics of binding of the ATM inhibitor are therefore an important factor, which would impact the pharmacokinetic profile for clinical candidates. These key factors are explored further in this chapter wherein the evolution of ATM inhibitors from tools to potential therapeutics is described.

Finally, single agent use, which could exploit potential synthetic lethal interactions, may require different inhibition kinetics and pharmacokinetic profiles for optimal activity. This has been well studied in the case of PARP inhibitors exploiting defects in homologous recombination repair (Bryant et al. 2005; Farmer et al. 2005; Kaelin 2005). It would remain to identify an appropriate patient population (and tumour signature), in whom ATM would cause such synthetic lethality but candidates arising from preclinical work include, APE1 and XRCC1 (Sultana et al. 2012, 2013), and potentially ATR, although it is inhibition of the latter that is reported as synthetic lethal with ATM loss (e.g. Reaper et al. 2011). It is worth noting that PARP inhibition is also synthetically lethal with loss of ATM (Aguilar-Quesada et al. 2007; Weston et al. 2010; Kubota et al. 2014), and this may provide an opportunity to combine two compounds to induce a tumour killing effect, an aspect that may be explored clinically with the appropriate molecules, as addressed at the end of this chapter.

In establishing the potential of ATM as a drug target, KU-55933 laid the foundations for the chemistry to follow, however, the utility of KU-55933 as a tool was limited by its poor physicochemical properties, in particular low aqueous solubility and low oral bioavailability. Whilst the in vitro data generated with the tools described was encouraging, the true nature of the effects on the broader biology may only be apparent when observed in vivo and in pre-clinical evaluation of a potential therapeutic. It was, therefore, apparent additional work was required to improve the physicochemical and pharmacokinetic properties to obtain ATM inhibitors suitable for in vivo assessment.

Optimization of KU-55933 resulted in the discovery of the second generation ATM inhibitors, KU-60019 (6) and KU-59403 (7), in which polar substituents have been appended to the tricyclic core resulting in improved potency and aqueous solubility (Batey et al. 2013; Golding et al. 2009). KU-60019 and KU-59403 are potent inhibitors of ATM enzyme (IC_{50} = 0.006 μM and 0.003 μM respectively), that retain high selectivity over ATR, DNA-PK, mTOR and PI3K (>270 fold and >300 fold respectively). Cellular potency was also improved relative to KU-55933 with KU-60019 and KU-59403 giving effective chemo-sensitisation at a concentrations of 3 μM and 1 μM respectively (concentrations of 10 μM were required in experiments using KU-55933). Cellular potency remains lower than in the biochemical assay, though this is typical of kinase inhibitors due to the target protein affinity for, and high cellular concentration of, ATP; typically a 100-fold drop off is observed for PIKK inhibitors (Knight and Shokat 2005). Although KU-60019 shows improved aqueous solubility when compared to KU-55933, it remains suboptimal for in vivo evaluation. However, by direct intracranial injection of the inhibitor into mice bearing orthotopically grown glioma tumours, in vivo radio-sensitisation was observed (Biddlestone-Thorpe et al. 2013). In this model, it was possible to demonstrate p53 independent inhibition of tumour growth to the point of achieving an apparent cure in some animals. Previous studies into radio-sensitisation of p53-deficient cells were performed in vitro (Teng et al. 2015), and may indicate a limitation of tools used only in vitro. Serendipitously, the use of an in vivo tool was able to unveil another aspect of ATM inhibitor biology.

Whilst the physicochemical and pharmacokinetic properties of KU-59403 were also found to be suboptimal for oral administration, further assessment of ATM inhibition in vivo was conducted with this compound following intraperitoneal (i.p.) injection. When dosed at 50 mg/kg i.p., tumour concentrations of KU-59403 above those required to deliver effective chemo- or radio-sensitisation in vitro were achieved for 4 h. Whilst these exposures may seem modest it has been reported that just transient inhibition of the target is sufficient to yield radio-potentiation (Rainey et al. 2008). Administration of KU-59403 alone did not result in any significant anti-tumour efficacy, consistent with a role as a sensitising agent, but when dosed at either 12.5 or 25 mg/kg i.p. BID for 5 days, sensitisation to cytotoxic therapy was observed. In combination with etoposide, a dose-dependent increase in tumour growth delay was observed in the colorectal tumour models, HCT-116 and SW620 (Batey et al. 2013). A similar dosing schedule combining KU-59403 with irinotecan was also shown to be effective without any obvious unacceptable adverse effects on the animals (as measured by body weight loss). The lack of overt toxicity from KU-59403 was important in establishing that an active ATM inhibitor would not result in inhibition of the target to the detriment of normal tissue, even in combination with cytotoxic therapy and, therefore, that an ATM inhibitor could be developed to enhance the therapeutic effect of chemotherapy (and by inference, radiotherapy). This observation reflected upon the suggestion that too prolonged an inhibition of ATM could be to the detriment of normal cellular processing of DNA damage as the lesion could remain unresolved on the DNA (Choi et al. 2010; White et al. 2010; Yamamoto et al. 2012). Importantly, additional experiments in cells lacking the expression of p53 would explore further the development of ATM inhibitors as clinical candidates. Activity was not compromised by loss of p53, consistent with observed radio-sensitisation that was more pronounced in cells with a p53 deficiency (Bracey et al. 1997; Powell et al. 1995; Yao et al. 1996). In fact, although Batey et al. (2013), reported equivalent activity in p53 deficient and proficient tumours, in another study, ATM inhibition resulted in increased efficacy in p53-mutated tumours (Biddlestone-Thorpe et al. 2013), and thus further selectivity towards tumours might be expected.

AZ32 (8) is a high affinity inhibitor of ATM enzyme ($IC_{50} < 0.006$ μM) discovered in the AstraZeneca laboratories following a focussed screening campaign. This resulted in novel chemistry that lacked the morpholine group that had been present in much of the earlier ATM chemistry, indicating that diversification was possible. AZ32 is a moderately potent inhibitor of ATM in cells ($IC_{50} = 0.31$ μM) but shows good selectivity over ATR in a cell based assay ($IC_{50} > 23$ μM). In addition to being potent and selective (only 4 of 124 kinases found to be inhibited >50% at 10 μM), AZ32 has been shown to be highly permeable in MDCK cell lines overexpressing MDR1 (P_{app} A-B = 33.7×10^{-6} cm/s, efflux ratio = 0.4) (Durant et al. 2016) and therefore brain penetrant, and has been shown to be orally bioavailable in rodents. An oral dose of 200 mg/kg in mice was shown to potentiate the effects of irinotecan in a xenograft model, consistent with the data obtained for KU-59403 (Batey et al. 2013). It is interesting to note that the physicochemical properties of AZ32, namely high permeability and low efflux, meant that when dosed at this level, unbound

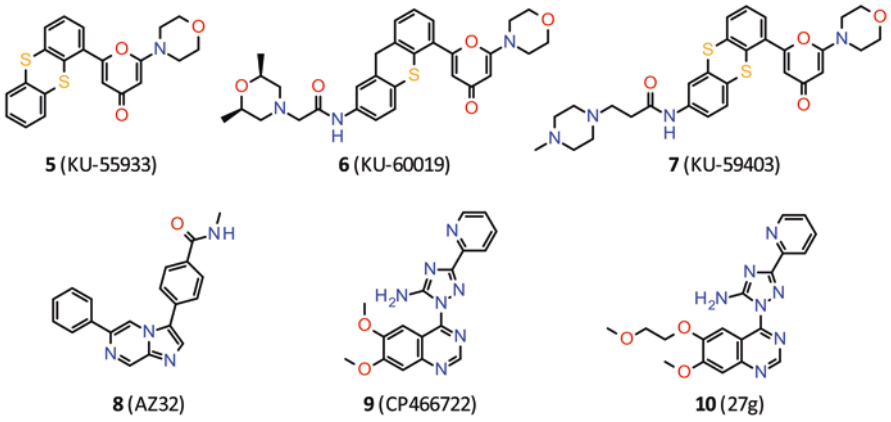


Fig. 8.2 Published structures of selective ATM inhibitors

concentrations in the brain exceeded the cellular IC_{50} for approximately 22 h. This level of ATM engagement in brain tissue would be anticipated to be beyond that required to deliver radiosensitisation. Similar levels of exposure where achieved with a 50 mg/kg oral dose of AZ32 utilising a more optimal formulation and, when combined with IR, this dose resulted in a significant increase in survival of mice bearing orthotopic gliomas (Durant et al. 2016). This result highlights the potential for brain penetrant ATM inhibitors to find therapeutic utility in the treatment of brain cancers (Fig. 8.2).

The quinazoline CP466722 (compound 9) was identified following a screen of 1500 compounds from the Pfizer compound library and shown to be a moderately potent inhibitor of ATM in cells (IC_{50} = 0.37 μM). CP466722 selectivity is not optimal and activity against a number of additional kinases was observed, including PIKK enzymes [additional significant activity against 106 out of 451 kinases was observed (tested at 3 μM)]. Importantly for use as a tool to explore the biology of ATM inhibition, little activity was observed against either ATR or DNA-PK in cells (Rainey et al. 2008), thereby allowing the observed radiosensitisation of HeLa cells to be interpreted as ATM dependent. CP466722 has low aqueous solubility (28 μM) and high clearance with a short half-life in mice (CL = 160 mL/h, $t_{1/2}$ = 1 h) and was, therefore, unsuited for further in vivo assessment. Optimization of the pharmacokinetic properties resulted in the identification of 27 g (compound 10) which, although being a less potent inhibitor of ATM (IC_{50} = 1.2 μM), benefited from an increase in selectivity [active against 41 out of 451 kinases (tested at 3 μM, though it should be noted this is only twofold selective over ATM)] and half-life in C57BL/6 mice ($t_{1/2}$ = 19 h) thus facilitating further in vivo evaluation. Despite an approximately fourfold enhancement of radiosensitivity for both CP466722 and 27 g in a clonogenic assay in which MCF7 cells were treated with 10 μM compound and irradiated with increasing doses of IR (0, 2 and 4 Gy), there have only been a small number of other publications reporting data on either compound. Indeed, one of the subsequent publications using CP466722 highlights the need for consideration of

the selectivity of the molecule as the potentiation of radiosensitivity observed in breast stem cells at 100 μM of CP466722 could have been caused by off target effects (Kim et al. 2012). An alternative approach using the molecule found that the inhibition of ATM by CP466722 (3 μM), mirrored that of KU-55933 (10 μM) with both molecules sensitising to the effects of temozolomide in a glioblastoma cell model (Nadkarni et al. 2012).

Whilst a range of structurally diverse ATM inhibitors had been identified, the identification of a molecule that combined high levels of potency and selectivity with good oral exposure and other drug like properties, remained elusive. It was appreciated that such a molecule would be required to truly interrogate the biology of ATM inhibition in vivo and to offer the potential for clinical utility. This need encouraged the continued search for novel ATM inhibitors and is described in the next section.

8.5 Evolution of In Vivo Active Probe Molecules

Although BEZ-235 was described earlier in the chapter as an in vivo and indeed clinically active compound, the molecule was classified with the other non-specific inhibitors of ATM and thus is not considered in the evolution of selective ATM inhibitors.

AstraZeneca set about a novel discovery program to identify alternative hit matter that could extend the utility of ATM inhibitors beyond that of the limitations described above. In a directed screen of approximately 15,000 compounds from an internal compound collection, compounds were identified with the ability to inhibit the phosphorylation of ATM on Serine 1981 in HT29 cells following irradiation. Analysis of the data generated identified the quinoline carboxamide hit (compound 11), as a moderately potent inhibitor of ATM in cells (IC_{50} = 0.82 μM), but importantly with selectivity for ATM over ATR (as measured by inhibition of pCHK1 in HT29 cells following treatment with 4-nitroquinoline 1-oxide (4NQO), (IC_{50} = 4.4 μM)). Compound 11 was shown to be a potent inhibitor of ATM enzyme (IC_{50} = 0.008 μM), with selectivity over closely related enzymes (>10-fold selective for ATM over DNA-PK and PI3Kα and >100-fold selective over mTOR, PI3Kβ and PI3Kγ). Compound 11 also showed encouraging selectivity when assessed against a diverse panel of kinases with only eight of the 124 kinases tested showing >50% inhibition when tested at 1 μM. Whilst representing a novel and potentially selective series of ATM inhibitor, it was appreciated that compound 11 shared similar sub-optimal physicochemical properties to many of the early ATM probe molecules. In particular, compound 11 had low aqueous solubility (19 μM), high intrinsic clearance (CL_{int}) in hepatocytes (Rat CL_{int} = 74 μL/min/10^6 cells, Human CL_{int} = 74 μL/min/10^6 cells), and activity against the hERG (human ether-a-go-go related gene) ion channel (IC_{50} = 2.3 μM) (Redfern et al. 2003; Waring et al. 2011); however, it was considered that the relatively high lipophilicity of this hit (log $D_{7.4}$ = 3.5), may in part, have been responsible for these properties. An optimization campaign was

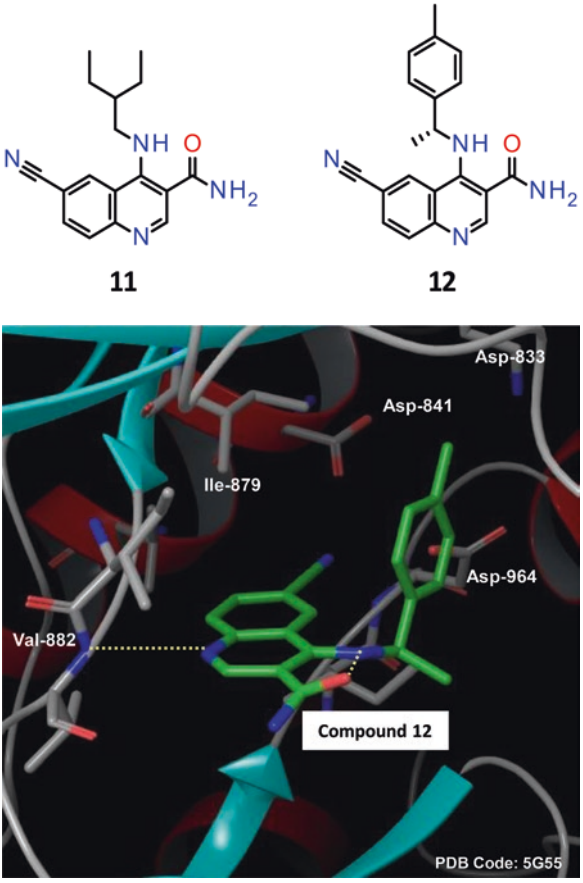


Fig. 8.3 (**a**) Structure of screening hit **11** and closely related PI3K inhibitor **12**. (**b**) X-ray structure of **12** bound into PI3Kγ (PDB code: 5G55)

subsequently initiated with the aim of improving both potency and selectivity whilst simultaneously optimising physicochemical and pharmacokinetic parameters (Degorce et al. 2016) (Fig. 8.3a).

Utilising a strategy similar to that described in the optimisation of KU-55933 the group within AstraZeneca were able to infer key features of the binding interaction between compound 11 and ATM by making an analogy with the interactions observed between a closely related structure, compound 12, and PI3Kγ (Yang et al. 2013) (Fig. 8.3b). Close inspection of the structure [Fig. 8.3b, PDB 5G55, (Degorce et al. 2016)], showed the quinoline nitrogen forming a key interaction with the hinge region of the kinase as well as suggesting the potential to further optimise the 6-cyano and 4-amino substituents. An internal hydrogen bonding interaction between the 4-amino substituent and the 3-carboxamide substituent can also be observed, presumably helping to organise the molecule in a bioactive conformation.

Although compound 12 does not have appreciable activity against ATM in cells ($IC_{50} > 30$ μM), it was hypothesised that a similar binding mode may be adopted by 11 when bound into ATM.

Exploration of the structure-activity relationship, SAR, for the compounds [detailed in (Degorce et al. 2016)], established that significant improvements in both ATM cellular potency and selectivity could be achieved by the introduction of an aromatic group in the 6-position, in particular a 3-pyridine motif. Additional SAR confirmed that small substituents could be introduced to the pyridine ring to further improve potency and selectivity. Parallel with the optimization of the 6-position, high throughput chemistry approaches were utilized to vary the 4-amino substituent with the intent of reducing the lipophilicity and improving physicochemical and pharmacokinetic properties. Opportunities to maintain potency and selectivity whilst improving other properties were identified by incorporating very specific chiral amines in the 4-position. When combined with the optimised substituents in the 6-position, highly potent and selective compounds were identified which showed excellent solubility and good oral bioavailability in rodent and dogs, for example compound 13 (AZ31) and compound 14, Fig. 8.4 [described as compound 74 by (Degorce et al. 2016)].

AZ31 and compound 14, are potent inhibitors of ATM enzyme ($IC_{50} < 0.0012$ μM and <0.0006 μM respectively), with excellent selectivity over closely related enzymes (>500 fold selective over DNA-PK and PI3Kα and >1000 fold selective over mTOR, PI3Kβ and PI3Kγ for both compounds). When tested against a diverse range of kinase targets, AZ31 inhibited 0 out of 126 kinases by >50% when tested at 1 μM, and compound 14 inhibited only 3 out of 386 kinases by >50% when tested at 1 μM. Assessment of permeability using Caco2 cells showed both compounds to be permeable (AZ31: P_{app} A-B = 5.2×10^{-6} cm/s; compound 14: P_{app} A-B = 14×10^{-6} cm/s) and in vivo pharmacokinetic evaluation showed both compounds to orally bioavailable in rat and dog (AZ31: F = 46% and 31% respectively; compound 14: F = 29% and 71% respectively). Furthermore, good exposure was observed following oral administration of both compounds to mice with doses of 100 mg/kg QD of AZ31 and 50 mg/kg BID of compound 14 giving unbound plasma exposures in excess of the ATM cell IC_{50} for approximately 24 h. The efficacy of AZ31 was assessed in HT29 tumour-bearing immunocompromised mice following oral administration at 100 mg/kg QD, in combination with IR (2 Gy delivered on

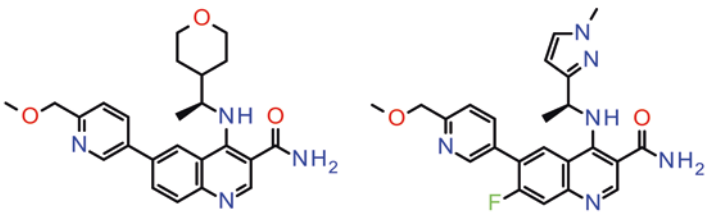


Fig. 8.4 Structure of AZ31 **13** and closely related inhibitor, compound **14**

each of days 1–5 of the study). Whilst AZ31 treatment alone did not reduce tumour growth and IR alone gave only a modest benefit, the combination of AZ31 and IR produced a significant reduction of tumour growth highlighting the radio-sensitising effect of ATM inhibition in this in vivo model. The ability for AZ31 to potentiate the effect of DNA BSB inducing chemotherapy was assessed in an SW620 (colorectal cancer cell line) xenograft model. Significant reduction in tumour growth was observed following oral administration of AZ31 at 100 mg/kg QD combined with irinotecan dosed at 50 mg/kg Q7D i.p. Interestingly, in this model tumours started to regrow following the cessation of treatment; however, retreating with the combination again led to tumour regression. No monotherapy effect was seen in this model for AZ31 and monotherapy efficacy for irinotecan was modest. No overt toxicity was observed in these studies and dosing was continued throughout the 21-day dosing period. In a GL261 glioma syngeneic and intracranial model, it was demonstrated that targeted delivery of ionising radiation combined with AZ31, resulted in enhanced therapeutic response compared to radiation alone but without morbidity or overt toxicity (Kahn et al. 2017). In contrast, treatment with ATM inhibitors alone had no therapeutic effect and combination of ATM inhibition with whole head irradiation resulted in mucositis and difficulty eating and drinking, suggestive normal tissue toxicity had occurred. AZ31 was also used in combination experiments with whole body irradiation of mice; combination of irradiation with AZ31 led to a reduced time to the mice becoming moribund and a more marked disruption of crypts leading to gastrointestinal syndrome (Vendetti et al. 2017). Enhancement of radiation induced toxicity in a murine model indicates that ATM inhibition is not restricted to tumour tissue but the data from Kahn et al. (2017), would suggest that targeting of radiation therapy may minimise the risk of toxicity to normal tissues for such combination therapies of ATM inhibitor and irradiation.

The efficacy of compound 14 was explored in SW620 tumour-bearing immunocompromised mice following oral administration at 50 mg/kg BID (dosed on days 2–4 of a weekly cycle) in combination with irinotecan dosed at 50 mg QD i.p. (dosed on day 1 of a weekly cycle). This combination schedule was tolerated and gave a significant tumour growth reduction following a 3 week regimen which was found to be statistically significant and greater than the reduction observed with irinotecan treatment alone (Degorce et al. 2016).

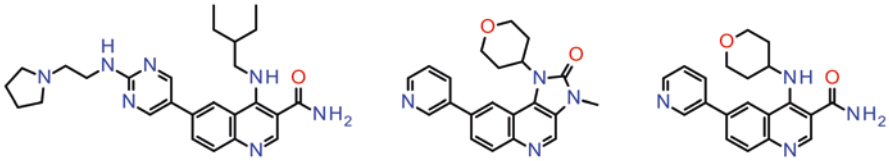
AZ31 and compound 14 have been demonstrated to be both efficacious and well tolerated and to give unbound exposures of ATM inhibitor in excess of both the enzyme and cell IC_{50}, following oral dosing. As discussed earlier, distinctions between ATM inhibition and kinase dead ATM or ATM loss should also be appreciated. The ability to administer the AZ31 and compound 14 in combination with chemo- and radiotherapy and determine combinatorial effects in the absence of single agent ATM inhibitor activity or toxicity was therefore an important observation and a step forward in the development of ATM inhibitors. However, whilst the potency, selectivity and preclinical pharmacokinetics for AZ31 and compound 14 appear encouraging, more detailed profiling suggested that these compounds were sub-optimal for consideration as a clinical candidate. In particular, AZ31 showed unwanted activity against the human-ether-a-go-go (hERG) potassium ion channel,

a known risk for adverse cardiovascular events, and both compounds were predicted to require relatively high doses to drive the desired level and duration of target engagement in the clinic. Detailed modelling of preclinical data suggested that neither AZ31 n or 14 would satisfy stringent criteria for clinical development (Ding et al. 2012; Hilgers et al. 2003; Johnson and Swindell 1996; Page 2016). These observations supported the continued optimisation of the compounds with a particular focus on reducing the predicted clinical dose whilst maintaining the otherwise promising properties. The further evolution of ATM inhibitors and the eventual development of a molecule with the attributes to be considered as a clinical candidate are described in the next section.

8.6 Clinical Candidate ATM Inhibitors

In developing ATM inhibitors to their ultimate clinical utility, it became necessary to substantially expand the medicinal chemistry effort and address critical issues of bioavailability and dosage, beyond the previous explorations of mechanism and feasibility of inhibition as a therapeutic strategy. When considering opportunities for the further optimisation of compounds such as AZ31 and 14, the optimisation of half-life was identified as a promising strategy to reduce the predicted clinical dose. The relatively low metabolic turnover of these compounds directed the strategy towards increasing the volume of distribution (V_{ss}) as a means to increase half-life. The importance of pK_a in determining V_{ss} has been long appreciated with basic compounds often showing considerably higher volumes than neutral and acidic compounds (Smith et al. 2015). Therefore, the opportunity to increase V_{ss}, and thereby half-life, through the incorporation of basic functionality was appreciated and adopted as an optimisation strategy.

Exploration of the 4- and 6-substituents of the quinoline carboxamide scaffold resulted in a wealth of data to aid SAR understanding. Review of these data identified that a basic substituent to support the enhanced V_{ss} strategy could be incorporated in the 6-position, as exemplified by compound 15, Fig. 8.5. Compound 15 is a highly potent and selective inhibitor of ATM in cells (IC_{50} = 0.0086 μM), with little or no activity against ATR (IC_{50} > 30 μM). Whilst providing evidence that basic functionality could be tolerated with respect to ATM binding, compound 15 was shown to possess a significantly compromised permeability profile (MDCK-MDR1



15 16 17

Fig. 8.5 Structures of **15**, **16** and **17**

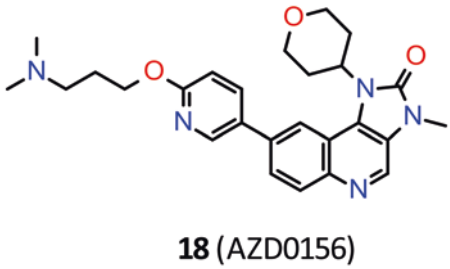
P_{app} A-B = 0.8 × 10^{-6} cm/s, efflux ratio = 28). Such a permeability profile was felt to limit the in vivo utility of the molecule and was considered to be driven primarily by a combination of both the basic functionality and the number of hydrogen bond donors present in the molecule. Whilst there remained an opportunity to increase permeability through the continued increase in lipophilicity, this strategy was not adopted due to the likely detrimental impact on many other key properties. Given the important role of both the 3-carboxamide and 4-amino motifs in the pre-organisation of the molecules into a bioactive conformation, the removal of either of these groups as a means to reduce hydrogen bond donor count was considered unlikely to succeed and attention was focussed on the identification of a more permeable scaffold.

The concept of utilising intramolecular hydrogen bonding interactions to constrain molecules in defined conformations is well known and has been used successfully in "scaffold hop" strategies where covalently bonded cyclic systems have been replaced with hydrogen bonded constrained acyclic systems (Furet et al. 2008). In the case of the quinoline carboxamide scaffold there already exists such a hydrogen bonded constrained acyclic system between the 4-amino substituent and the 3-carboxamide substituent suggesting that replacement with a covalently bonded cyclic system may be feasible. Such an approach would result in a significant reduction in hydrogen bond donors. The feasibility of this approach was confirmed by the synthesis of the imidazo[5,4-c]quinolin-2-one containing compound 16 (Fig. 8.5). Indeed, the dual PI3K/mTOR inhibitor NVP-BEZ235 (compound 4), described earlier, contains this same imidazo[5,4-c]quinolin-2-one scaffold, thus providing further evidence of the ability of this scaffold to inhibit ATM, whilst simultaneously highlighting the challenge of achieving the required level of selectivity, not apparent in the earlier example.

Comparison of quinoline carboxamide (17) with the analogous imidazo[5,4-c] quinolin-2-one (16), shows that ATM potency is broadly maintained (17: ATM cell IC_{50} = 0.95 μM, 16: ATM cell IC_{50} = 0.36 μM); however, the selectivity of compound 16 against closely related kinases was significantly reduced and indeed 16 was shown to have greater affinity for ATR than for ATM (16: ATR cell IC_{50} = 0.087 μM). Imidazo[5,4-c]quinolin-2-one,16, did show the anticipated increase in permeability and reduction in efflux compared to quinoline carboxamide analogue 17. This improved permeability was achieved with only a modest increase in lipophilicity (Δ log $D_{7.4}$ = 0.3), supporting the hypothesis that the imidazo[5,4-c] quinolin-2-one is an inherently more permeable scaffold and that this is predominantly driven by reduced number of hydrogen bond donors.

With the identification of a more permeable scaffold attention was once again turned to the incorporation of basic functionality to drive increased V_{ss} and half-life. Significant optimisation of the basic substituent delivered not only improved properties and pharmacokinetics but also delivered a dramatic improvement in ATM affinity and selectivity, as exemplified by compound 18, subsequently known as AZD0156, Fig. 8.6.

AZD0156 is an exceptionally potent inhibitor of ATM, with over 1000-fold improvement on the first generation selective inhibitors such as KU-55933 and



18 (AZD0156)

Fig. 8.6 Structure of screening AZD0156 (**18**)

Table 8.1 Potency and selectivity data for AZD0156

Target	Enzyme IC_{50} (μM)	Cell IC_{50} (μM)
ATM	0.00004[a]	0.00058
ATR	–	6.2
DNA-PK	0.14	–
mTOR	0.20	0.61
PI3Kα	0.32	1.4
PI3Kβ	1.8	–
PI3Kγ	1.1	–
PI3Kδ	0.27	–

[a]IC_{50} value corrected for tight binding

Table 8.2 Physicochemical and pharmacokinetic properties of AZD0156

	AZD0156
Crystalline solubility	>800 μM
% free (rat, dog, human)	11.4%, 40.9%, 29.0%
MDCK P_{app} A–B/efflux ratio	6.6×10^{-6} cm/s/5.1
Caco2 P_{app} A–B/efflux ratio	5.6×10^{-6} cm/s/8.5
Hepatocyte CL_{int} (rat, dog, human)	3.3, 3.3, 5.7 μL/min/10^6 cells
Rat PK (CL, V_{ss}, F)	15.5 mL/min/kg, 4.3 L/kg, 57%
Dog PK (CL, V_{ss}, F)	33.3 mL/min/kg, 17.6 L/kg, 54%
CYP inhibition (3A4, 2D6, 2C9, 1A2, 2C19)	$IC_{50} > 30$ μM

retaining excellent selectivity over closely related targets in both enzyme and cell based assays, Table 8.1.

When screened at 1 μM against a panel of 397 kinases AZD0156 showed excellent general kinome selectivity with activity above 65% inhibition observed for only 5 kinases (HASPIN: 67%; JAK1 (JH2domain-pseudokinase): 67%; LRRK2: 87%; mTOR: 93%; PIK4CB: 70%). A stable crystalline form of AZD0156 was identified and shown to have good aqueous solubility. AZD0156 has high levels of unbound drug in rat, dog and human plasma, is permeable with good pharmacokinetics in both rat and dog, and does not inhibit any of the five major isoforms of human cytochrome p450 at the concentrations tested (Table 8.2). AZD0156 was predicted to have a low clinically efficacious dose (<10 mg) based on preclinical models and as such was considered suitable for clinical development.

AZD0156 has evolved to become the ultimate tool for ATM inhibition allowing the potential to explore the inhibition of ATM in a clinical setting. In order to support positioning of the compound in the clinic, the ability of AZD0156 to potentiate the efficacy of DNA damage inducing agents was assessed in vitro by combining with either SN-38 (the active agent of the topoisomerase I inhibitor irinotecan) or the PARP inhibitor olaparib. AZD0156 shows good exposure in mice thereby allowing the in vivo assessment of ATM inhibition in mouse xenograft models, at tolerated intermittent schedules with chemotherapy or olaparib. When combined with irinotecan dosed at 50 mg/kg i.p (on day 1 of a weekly cycle), AZD0156 dosed orally at 20 mg/kg QD (on days 2–4 of a weekly cycle) showed clear synergy and caused tumour regression in an SW620 xenograft model. No appreciable efficacy was observed in this model when AZD0156 was dosed as a monotherapy. The addition of AZD0156, dosed orally at 5 mg/kg QD (on days 1–3 of a weekly cycle), to olaparib, dosed orally at 50 mg/kg QD, also resulted in clear synergy and tumour regression when examined in mice bearing an BRCA-2 mutant TNBC patient derived tumour (Pike et al. submitted).

The drug like qualities of AZD0156 has enabled toxicological assessment in both rat and dog and AZD0156 has entered clinical evaluation in a Phase I clinical trial, alone and in combination with olaparib (detailed on clinicaltrials.gov; NCT02588105). The preclinical data support the tolerability of the combination of AZD0156 and olaparib with no interruption of dosing nor overt toxicity observed (no body weight loss) in immune compromised mice harbouring an BRCA2 mutated patient derived xenograft. The clinical studies will establish both the pharmacokinetics of AZD0156 (single agent studies) and tolerated doses for the single agent and combination dosing.

One further opportunity has arisen for the development of ATM inhibitors (Golding et al. 2012). Earlier in the chapter, it was highlighted that brain penetrant nature of AZ32 allows for unbound drug levels in brain tissue to exceed the IC_{50} for ATM inhibition for a sustained period following oral administration. Treatment for Glioblastoma Multiforme (GBM), involves surgery followed by fractionated radiotherapy and temozolomide which provides a median survival of just 12–15 months (Ajaz et al. 2014; Delgado-López and Corrales-García 2016). Poor survival is attributed to an inability to excise all invasive tumour tissue (if operable) and an intrinsic tumour chemo/radioresistance. Equally challenging, is the current poor prognosis of patients with primary malignancies that metastasise to the brain. Single or multiple brain metastases are also refractory to current chemo/radiotherapy regimes and usually signifies end-stage disease (Lin and DeAngelis 2015). One third of GBM tumours contain p53 mutations and ~80% harbour other cell-cycle checkpoint alterations and it has been shown that p53-defective GBM cells are much more radiosensitised that wildtype cells (Roy et al. 2006; Biddlestone-Thorpe et al. 2013; Durant et al. 2016). In addition, reports have shown ATM knock-out mice brains are actually protected from acute adverse effects of radiation and that ATM promotes radiation induced apoptosis in post-mitotic, neural stem cell (NSC) (Gosink et al. 1999; Herzog et al. 1998), and in subventricular zone cells after low doses of radiation; NSC populations in ATM deficient embryos and adult mice

exhibit radioresistance (Barazzuol et al. 2015; Gatz et al. 2011). All these studies may suggest that a potentially wide therapeutic window may exist between normal and brain tumour tissue. Furthermore, in assessing CP466722, Nadkarni et al. (2012), determined that glioblastoma cells sensitive to temozolomide could be further sensitised by combination with ATM inhibition as a route to achieving a greater impact on tumour growth inhibition through combination therapy.

AstraZeneca disclosed AZ32 at the 2016 AACR Annual Meeting (Durant et al. 2016), as a specific inhibitor of ATM possessing good blood-brain barrier (BBB) penetration in mouse. Based on these data, AstraZeneca continues to invest in developing a BBB-penetrating ATM inhibitor for clinical use in combination with radiotherapy for the treatment of primary malignancies of the brain and CNS as well as brain metastases. To this end, further optimisation of a molecule active in mice has resulted in the discovery of AZD1390, a potent and selective ATM inhibitor which is anticipated to efficiently cross the BBB in man. AZD1390 affords excellent efficacy in preclinical orthotopic brain tumour models and represents an exciting addition to the candidate drug portfolio for ATM inhibition. The structure of AZD1390 has yet to be disclosed but as clinical development continues then more information on this agent is expected to be released.

8.7 Concluding Remarks

Early molecules that enabled exploration of the in vivo activity of ATM inhibitors and the potential for chemo- and radio-sensitisation of tumour cells, were flawed and failed with poor selectivity. The improvement in selectivity and design of ATM specific inhibitors such as KU-55933, substantially improved the understanding of the in vitro properties, phenotypes and complex biology of ATM inhibition but were compromised by poor potency, selectivity or pharmacokinetic properties that restricted their utility as in vivo probes. However, in the past decade, a number of substantial improvements have been made and the availability of molecules, including AZ31 and Compound 14, have enabled both selective inhibition of ATM and exploration of the in vivo consequence of ATM inhibition, enhancement of tumour killing from combination therapies and a lack of obvious toxicity to normal tissues. In the context of combination with chemotherapy, it is perhaps the transient nature of ATM inhibition that provides a therapeutic window between the killing of tumour cells and normal cell toxicity – intermittent scheduling of chemotherapy or olaparib with AZD0156 were shown to be tolerated and efficacious in vivo suggesting scheduling may also result in clinically tolerated combinations. Together with the inherent sensitivity of genetically unstable tumours, that may lack e.g. p53 could further separate tumour sensitivity as it would not be constrained by the checkpoints and DNA damage responses afforded to normal tissue. In the case of combinations with irradiation, the exposure of normal tissue to ionising radiation in the presence of ATM inhibitors may be more toxic than radiation alone (Kahn et al. 2017; Vendetti et al. 2017), but targeted delivery of therapy may avoid such collateral toxicity (Kahn et al. 2017). In addition, studies in ATM knock-out mice have shown normal

brain tissue to be relatively radio-resistant, in fact, potentially protected from the effects of radiation compared with wild type mice, suggesting a wide therapeutic window may exist particularly in brain.

Further improvements to increase pharmacokinetic half-life and thereby reduce clinical dose has resulted in the discovery of AZD0156, an extremely potent and selective ATM inhibitor with good physicochemical properties and preclinical pharmacokinetics. AZD0156 has allowed more detailed in vivo target validation and has now entered clinical trials. The initial stages of the clinical evaluation of AZD0156 will be to establish a tolerated dose as a single agent and in combination with olaparib and with cytotoxic chemotherapies, in a typical Phase I patient population of patients with advanced disease in a range of tumour indications. Secondary endpoints will determine if biomarker changes and enhancement of therapeutic effect of PARP inhibition or combination therapies can be detected in circulating tumours cells or circulating tumour DNA. One of the key challenges facing the successful clinical development of ATM inhibitors will be to understand how best to combine with the variety of DSB inducing agents, and which patients will respond best to such combinations. The earlier molecules and the data generated with these can help guide such combinations, but as the use of AZD0156 further develops our understanding of the biology and potential of ATM inhibition, additional opportunities for rational combinations or even for monotherapy treatment may well emerge (Morgado-Palacin et al. 2016).

The potential for ATM inhibition to combine with radiotherapy is also an area of active research and the importance of the blood-brain barrier in the potential to treat patients with brain tumours (such as GBM), will need to be established to ensure that clinical agents with suitable profiles can be developed. The biology of ATM is known to include its activation by ROS and the emergence of high quality ATM inhibitors will allow more detailed investigations into additional therapeutic areas, potentially beyond cancer. Early work with KU-55933 gave evidence to a role in the inhibition of HIV infection (Lau et al. 2005), the virus requires host cell mechanisms to integrate into the genome and prevention of homologous recombination by inhibition of ATM prevents viral propagation. More recently, it has been shown that ATM inhibition may have therapeutic potential in Huntingdon's disease due to alleviation of persistent and elevated activation of ATM by the mutant Huntingtin protein (Lu et al. 2014). Both approaches are still anchored in the role of ATM in homologous recombination repair but show scope well beyond the role of the target in cancer biology. The ultimate scope of therapeutic ATM inhibition awaits further experimentation, enabled by the chemistry described here.

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