Transient Inhibition of ATM Kinase Is Sufficient to Enhance Cellular Sensitivity to Ionizing Radiation

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Abstract

In response to DNA damage, the ATM protein kinase activates signal transduction pathways essential for coordinating cell cycle progression with DNA repair. In the human disease ataxia-telangiectasia, mutation of the ATM gene results in multiple cellular defects, including enhanced sensitivity to ionizing radiation (IR). This phenotype highlights ATM as a potential target for novel inhibitors that could be used to enhance tumor cell sensitivity to radiotherapy. A targeted compound library was screened for potential inhibitors of the ATM kinase, and CP466722 was identified. The compound is nontoxic and does not inhibit phosphatidylinositol 3-kinase (PI3K) or PI3K-like protein kinase family members in cells. CP466722 inhibited cellular ATM- dependent phosphorylation events and disruption of ATM function resulted in characteristic cell cycle checkpoint defects. Inhibition of cellular ATM kinase activity was rapidly and completely reversed by removing CP466722. Interestingly, clonogenic survival assays showed that transient inhibition of ATM is sufficient to sensitize cells to IR and suggests that therapeutic radiosensitization may only require ATM inhibition for short periods of time. The ability of CP466722 to rapidly and reversibly regulate ATM activity provides a new tool to ask questions about ATM function that could not easily be addressed using genetic models or RNA interference technologies. [Cancer Res 2008; 68(18):7466–74]

Introduction

Cells respond to DNA damage by activating a complex network of signal transduction pathways. These DNA damage response (DDR) pathways include sensors responsible for recognizing the genotoxic insult, transducers responsible for relaying/amplifying the signal, and effectors that induce the appropriate cellular response. Together, these signaling cascades are responsible for coordinating cell cycle progression with DNA repair to facilitate maintenance of genomic stability (1).

The human autosomal recessive disease ataxia-telangiectasia (A-T) has a complex clinical phenotype, including progressive cerebellar ataxia, oculocutaneous telangiectasias, immune deficiency, hypogonadism, growth retardation, premature aging, radiosensitivity, and cancer predisposition (2). Cells obtained from A-T patients display DNA damage checkpoint defects in G1, S, and G2 phases of the cell cycle, increased chromosomal instability, and radiosensitivity (3). The defective gene in A-T was identified as ATM (A-T, mutated) and encodes a 350-kDa protein that belongs to the phosphatidylinositol 3-kinase (PI3K) family of proteins (4). Based on the phenotype displayed by A-T cells, it is not surprising that the ATM protein kinase has been characterized as a major regulator of the DDR pathways, along with the closely related family members ATR (A-T and Rad3-related kinase) and DNA-PK (DNA-dependent protein kinase; ref. 5). In an unperturbed cell, ATM exists as an inactive dimer (or higher-order oligomer), but the introduction of DNA double-strand breaks (DSB) by ionizing radiation (IR) or other insults activates the ATM kinase by intermolecular autophosphorylation and dimer dissociation (6, 7). Once activated, ATM phosphorylates several downstream substrates that contribute to the proper regulation of IR-induced arrests in G1 phase (e.g., p53, Mdm2, and Chk2; refs. 8–12), S phase (e.g., Nbs1, Smc1, Brca1, and FancD2; refs. 13–16), and G2 phase (e.g., Brca1 and Rad17; refs. 16, 17) of the cell cycle.

Studies of cells that are functionally defective in different components of the DDR pathways (e.g., BRCA1/2, NBS1, ATM, Mre11, Fanconi anemia proteins, etc.) show cell cycle checkpoint defects, decreased ability to repair damaged DNA, and an increased sensitivity to IR and other DNA damaging agents (2, 15, 18, 19). This latter observation highlights components of these DDR pathways as potential therapeutic targets for the development of small molecule inhibitors that could enhance the sensitivity of tumor cells to the cytotoxic effects of radiotherapeutic/chemotherapeutic agents (20–22).

The idea of using small molecule inhibitors to disrupt ATM function and sensitize tumor cells to radiotherapeutic/chemotherapeutic agents is not a novel concept (20–23). However, the most commonly used ATM inhibitors (caffeine and wortmannin) are neither specific nor useful in vivo, which has fueled an interest in identifying more specific and potent inhibitors and resulted in the recent identification of KU55933 (24). Using an in vitro kinase assay, we screened a targeted library of ~1,500 small molecule compounds for potential ATM inhibitors and identified CP466722. This compound inhibited ATM kinase activity in vitro, but did not inhibit PI3K or closely related PI3K-like protein kinase (PIKK) family members. The compound also inhibited the ATM signal transduction pathway in cells, disrupted cell cycle checkpoint function, and sensitized tumor cells to IR. CP466722 is a rapidly reversible inhibitor of ATM function, and transient exposure used in clonogenic survival assays suggests that short-term inhibition of ATM function is sufficient to sensitize cells to IR. This observation has potential implications for sensitization of tumor cells in vivo, where drug pharmacokinetics becomes an important consideration. Identification of CP466722 provides a novel chemical structure that inhibits ATM function in cells and can now be modified to generate more potent and specific agents that could be effective at enhancing tumor cell killing in vivo.
In addition, the fact that ATM function can be rapidly turned on and off provides new opportunities for studying the ATM pathway.

Materials and Methods

Chemicals

Pfizer identified CP466722 [2-(6,7-dimethoxyquinazolin-4-yl)-5-(pyridin-2-yl)-2H-1,2,4-triazol-3-amine]. CP466722 (3 mmol/L), KU55933 and wortmannin (10 mmol/L; Sigma) were resuspended in DMSO. Caffeine (10 mmol/L; Sigma) was resuspended in dH2O. Aphidicolin (5 mmol/L; Sigma) was resuspended in methanol. Recombinant human insulin-like growth factor-I (IGF-I; 0.01 mg/ml; Invitrogen) was diluted in dH2O [0.1% w/v bovine serum albumin (BSA)]. Cells were routinely pretreated with DMSO (vehicle control), CP466722 or wortmannin (30 min), and caffeine or KU55933 (1 h).

Cell Culture

Cells were plated (0.5 × 10⁶ per plate) 24 h before treatment and maintained at 37°C in a humidified atmosphere (5% CO₂). HeLa, normal diploid HFF, Mcf7 (American Type Culture Collection), HFF (expressing hTERT; ref. 25), and A−T (GM02052 expressing hTERT; ref. 26) cells were cultured in DMEM [10% v/v fetal bovine serum (FBS), 10 mmol/L HEPES, penicillin 100 units/ml, and streptomycin 100 μg/ml]. Atl-tyrpe and deficient mouse embryonic fibroblasts (MEF; Arf-deficient background; ref. 27) were cultured in DMEM (10% v/v FBS, 2 mmol/L glutamine, 0.1 mmol/L NEAA, 55 μmol/L L-mercaptoethanol, penicillin 100 units/ml, and streptomycin 100 μg/ml). Arf-deficient mouse pre-B cells expressing the human p185-BCR-ABL isoform (p185+/-) were cultured in DMEM (10% FBS, 2 mmol/L glutamine, 0.1 mmol/L NEAA, 55 μmol/L L-mercaptoethanol, penicillin 100 units/ml, and streptomycin 100 μg/ml). MEF-tyrpe and deficient mouse embryonic fibroblasts (MEF; Arf-deficient background; ref. 27) were cultured in DMEM (10% v/v FBS, 2 mmol/L glutamine, 0.1 mmol/L NEAA, 55 μmol/L L-mercaptoethanol, penicillin 100 units/ml, and streptomycin 100 μg/ml).

Cell viability. Cells were plated in triplicate (40,000 per plate) and incubated as required before culture media, trypsinized cells were combined, and viability was determined by Vi-CELL XR cell viability analyzer (Beckman Coulter).

Serum starvation and IGF-I stimulation. Cells were plated as normal, incubated for 24 h before being removed from culture media, washed with, and then cultured for 24 h in normal or low serum DMEM (0.1% v/v FBS, 10 mmol/L HEPES, penicillin 100 units/ml, and streptomycin 100 μg/ml). Cells were stimulated by addition of IGF-I (10 ng/ml) for 20 min at 37°C before harvesting.

In vitro Kinase Assays

To screen for small molecule inhibitors of ATM kinase activity, an in vitro kinase assay was adapted (10, 29) and an ELISA assay was developed which measured the phosphorylation status of the ATM downstream target p53. Recombinant glutathione S-transferase (GST)–p53(1-101) and full-length flag-tagged ATM and ATR were purified for use in the ELISA and in vitro kinase assays. Briefly, Nunc 96-well Maxisorp plates were coated overnight (4°C) with 2 μg of purified recombinant GST-p53(1-101) in PBS. All subsequent incubations were performed at room temperature. The plates were washed (0.05% v/v Tween/PBS) before addition of purified recombinant full-length ATM kinase (30–60 ng) in a final volume of 80 μL of reaction buffer (20 mmol/L HEPES, 50 mmol/L NaCl, 10 mmol/L MgCl₂, 10 mmol/L MnCl₂, 1 mmol/L DTT, and 1 μmol/L ATP) in the presence or absence of compound. Compounds (10 μM) were added to plates in duplicate, and the kinase assay was incubated (90 min). Plates were washed (0.05% v/v Tween/PBS) blocked (1 h, 1% w/v BSA/PBS), and rinsed before anti–phosphorylated (Ser1981) ATM (6). ImageJ3 was used to quantitate band density on autoradiograms from Western blotting, and relative inhibition was calculated as percentage of control.

Flow Cytometric Analysis

Cell cycle analysis. Cells were harvested and fixed (4°C) with 70% v/v ethanol-PBS. Cells (1 × 10⁶) were washed (PBS) and incubated (30 min/dark) at room temperature in PBS (10 μg/ml propidium iodide (Sigma), 250 μg/ml RNaseA (Qiagen)). DNA content was determined using a FACScan (Becton Dickinson), and data were analyzed (CellQuest software).

Immunofluorescent detection of phosphorylated histone H3. Cells were harvested 1 h after IR and fixed (−20°C) with 70% v/v ethanol-PBS. Cells were stained and analyzed as previously described (31).

Clonogenic Survival Assay

HeLa or A−T (GM02052 expressing hTERT) cells were plated in triplicate (0.5 × 10⁶ per plate) and incubated for 24 h. Cells were pretreated: DMSO, CP466722, or KU55933 before IR (0–10 Gy). Cells were incubated for 4 h after IR before media was removed, and cells were washed (PBS), trypsinized, counted, replated (2,000 per plate, 10-cm plates) in the absence of drug, and incubated for 10 d. Before colony counting, cells were washed (PBS), stained (PBS, 0.0037% v/v formaldehyde, 0.1% w/v crystal violet), rinsed (dH₂O), and dried. Defined populations (±40 cells) were counted as one surviving colony; data were calculated as percentage surviving colonies relative to control plates ± SE.

Results

Identification of an in vitro inhibitor of the ATM kinase.

Large amounts of purified protein would be required to run high throughput screens to identify small molecule inhibitors of ATM. Therefore, a directed screen-based approach was adopted where a library of 1,500 compounds was selected based on known kinase inhibitor templates and calculated kinase pharmacophores from the Pfizer proprietary chemical file. These compounds were screened using an in vitro ELISA assay, with potential inhibitors being identified by a decreased ability of purified ATM kinase to phosphorylate GST-p53(1-101) substrate (data not shown). Compounds identified by this assay were subjected to an in vitro kinase assay to screen out false positives (data not shown). This screening approach identified the compound CP466722 (Fig. 1) as a candidate

3 http://rsh.info.nih.gov/ij/
Chemical structure of CP466722 [2-(6,7-dimethoxyquinazolin-4-yl)-5-(pyridin-2-yl)-2H-1,2,4-triazol-3-amine].

Figure 1. Chemical structure of CP466722 [2-(6,7-dimethoxyquinazolin-4-yl)-5-(pyridin-2-yl)-2H-1,2,4-triazol-3-amine].

Figure 2. CP466722 inhibits ATM kinase activity in cells in response to IR-induced DNA damage. HeLa cells were preincubated with varying concentrations of CP466722, DMSO, or 10 μmol/L KU55933 (KU) before mock-IR (Control) or IR (2 Gy) followed by incubation at 37°C for 30 min before being harvested. To determine the effect of CP466722 on ATM kinase activity, ATM intermolecular autophosphorylation at Ser1981 and phosphorylation of downstream ATM targets were monitored by Western blotting analysis (representative of several repeat experiments).
cells lacking ATM provided even more definitive evidence that CP466722 does not inhibit ATR kinase in cells (Fig. 3A).

DNA-PK is another PIKK family member that contributes to damage-induced signaling, and both ATM and DNA-PK can phosphorylate histone H2AX on Ser 139 after IR (37). To investigate potential effects of CP466722 on DNA-PK, phosphorylation of histone H2AX (Ser139) was monitored by Western blotting analysis. Whereas H2AX (Ser139) phosphorylation after IR was inhibited by CP466722 or KU55933 in wild-type cells, these ATM inhibitors failed to inhibit IR-induced H2AX phosphorylation in A-T cells (Fig. 3B), demonstrating a lack of detectable effects on DNA-PK.

In response to growth factor stimulation, AKT is activated by phosphorylation of Thr308 by the PI3K pathway and Ser473 by other PIKK family members (e.g., mTOR, DNA-PK, and ATM; ref. 38). To show that CP466722 was not inhibiting PI3K or PIKK family members, human fibroblasts were serum starved for 24 hours before being stimulated with IGF-I either in the presence or absence of CP466722, KU55933, or wortmannin (Fig. 3C). Serum starvation resulted in an almost complete loss of AKT (Thr308/Ser473) phosphorylation. These phosphorylation events were strongly induced upon addition of IGF-I to serum-starved cells and, as expected, strongly inhibited by the known PI3K inhibitor wortmannin. No inhibition was noted with CP466722 or KU55933 treatment. Taken together, these results indicate that CP466722 inhibits ATM kinase, but does not affect the cellular activity of PI3K or PIKK family members.

Abl and Src kinases were identified in the initial in vitro screens as potential targets of CP466722. To address whether CP466722 inhibits cellular Abl and Src kinases, we used a mouse pre-B cell (p185+/+Arf−/−) model (28). In this system, the BCR-Abl

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**Figure 3.** PI3K and PIKK family members are not inhibited by CP466722 in cells. HFF(hTERT) and A-T(hTERT) fibroblasts were used to establish the effects of CP466722 on PI3K and PIKK family members. A, cells were preincubated with DMSO or 6 μmol/L of CP466722 (CP) before being mock-treated or treated with 20 μmol/L aphidicolin (Aph) for a 1-h period at 37 °C before harvesting. As a control, cells were preincubated with DMSO or 6 μmol/L of CP466722 before being exposed to IR (2 Gy) followed by incubation at 37 °C for 30 min and harvesting. To determine the effect of CP466722 on the ATM and ATR checkpoint pathways in response to aphidicolin and IR, phosphorylated (Ser345) Chk1 and phosphorylated (Thr68) Chk2 were monitored by Western blotting analysis. B, cells were preincubated with DMSO, 6 μmol/L CP466722 (CP), or 10 μmol/L KU55933 (KU) before mock-IR (Control) or IR (2 Gy) followed by incubation at 37 °C for 30 min and harvesting. As a measure of ATM/DNA-PK kinase activity, phosphorylated (Ser139) H2AX was monitored by Western blotting analysis. C, cells were cultured under normal (Control) or serum-starved (SS) conditions for a 24-h period before being preincubated with DMSO, 6 μmol/L CP466722 (CP), 10 μmol/L KU55933 (KU), or 200 nmol/L wortmannin (W). Cells were then subjected to growth factor stimulation with IGF-I (10 ng/mL) for a 20-min period at 37 °C before being harvested. The activity of PI3K and PIKK family members were assessed by monitoring phosphorylated (Thr308) Akt and phosphorylated (Ser473) Akt by Western blotting analysis (representative of several repeat experiments).
(p185) fusion protein is constitutively active, driving autophosphorylation of residue Tyr245 (BCR-Abl and endogenous Abl) and phosphorylation of a downstream target CrkL on Tyr207 (Supplementary Fig. S4A; refs. 39–41). Src kinase undergoes intermolecular autophosphorylation of residue Tyr416 on its activation loop to become fully activated (42, 43). In cells expressing BCR-Abl, SRC kinases are activated, and increased levels of Src (Tyr416) phosphorylation have been reported, suggesting that Src is active and undergoing autophosphorylation (Supplementary Fig. S4A; refs. 41, 44).

As a control, CP466722 and KU55933 were shown to inhibit ATM kinase activity in the mouse pre-B cells as shown by disruption of p53 (Ser15) phosphorylation and p53 stabilization in response to IR (Supplementary Fig. S4B). To establish whether the inhibitors affected Abl and Src kinase activity, the mouse pre-B cells were treated with CP466722, KU55933, or imatinib (Abl kinase inhibitor) as a positive control (45). As expected, autophosphorylation of BCR-Abl (Tyr245), endogenous Abl (Tyr245), and Abl-dependent phosphorylation of CrkL (Tyr207) were all detected in control mouse pre-B cells (Supplementary Fig. S4A). Imatinib inhibited all these phosphorylation events, whereas CP466722 or KU55933 failed to inhibit BCR-Abl kinase activity or phosphorylation of downstream targets. Although imatinib is not reported to directly inhibit Src kinase activity, cellular Src (Tyr416) autophosphorylation was prevented by imatinib under these experimental conditions (41, 45, 46). Treatment with both CP466722 and KU55933 resulted in decreased Src (Tyr416) autophosphorylation relative to the control cells (Supplementary Fig. S4A). These data indicate that at doses capable of inhibiting ATM, CP466722 and KU55933 do not inhibit Abl kinase activity in cells; however, both compounds have inhibitory effects on Src kinase activity in this system (84% and 73% inhibition at 10 μmol/L, respectively).

CP466722 disrupts ATM-dependent cell cycle checkpoints in cells. Small molecule disruption of the ATM signal transduction pathway should recapitulate the A-T cellular phenotypes, including characteristic cell cycle checkpoint defects. Cells lacking ATM exhibit pronounced G2 accumulation over time after IR due to a failure to arrest in S phase (31). In response to IR, HeLa cells treated with either KU55933 or CP466722 resulted in an enhanced proportion of cells with G2-M DNA content and a decreased proportion of cells with G1-phase DNA content relative to DMSO-treated cells (Fig. 4A). In the absence of IR-induced DNA damage, these doses of CP466722 and KU55933 had no effect on cell cycle distribution during this time frame (Fig. 4A, i and ii).

To establish whether CP466722 and KU55933 treatment disrupted the ATM-dependent G2-M checkpoint (31), asynchronous populations of HeLa cells were pretreated with either DMSO, caffeine, CP466722, or KU55933 before being exposed to mock-IR or IR. A decrease in the percentage of mitotic cells after IR in the presence of DMSO indicated an IR-induced G2 arrest, whereas both KU55933 and CP466722 prevented this IR-induced decrease (Fig. 4B). In contrast to the effects seen with the less specific ATM/ATR inhibitor, caffeine, neither compound affected G2-M progression in the absence of DNA damage (Fig. 4B).

Figure 4. CP466722 inhibits ATM function in response to IR-induced DNA damage. A, an asynchronous population of HeLa cells was preincubated with DMSO, 6 μmol/L CP466722, or 10 μmol/L KU55933 before mock-IR or IR (5 Gy). After irradiation, cells were incubated at 37°C for 16 h before being harvested, fixed, and stained with propidium iodide for cell cycle analysis by flow cytometry. i, DNA content profiles are representative of several repeat experiments. ii, data displayed by the DNA content profiles were analyzed and the cell cycle phase information is represented graphically. B, an asynchronous population of HeLa cells was preincubated with DMSO, 6 μmol/L CP466722, 2 mmol/L caffeine, or 10 μmol/L KU55933 before mock-IR or IR (2 Gy). After irradiation, cells were incubated at 37°C for 1 h before being harvested, fixed, and stained for phosphorylated (Ser10) histone H3 and propidium iodide. DNA content and phosphorylated (Ser10) histone H3 positivity were determined by flow cytometry, and the data displayed are representative of several repeat experiments.
Taken together, the results show that CP466722 is capable of disrupting ATM function and recapitulates checkpoint defects reported for A-T cells.

**Chemical inhibition of ATM can be rapidly and completely reversed.** KU55933 displays strong inhibition of ATM for at least 4 hours in tissue culture (24). To determine whether CP466722 could inhibit ATM for prolonged periods of time in tissue culture, HeLa cells were preincubated with DMSO, 6 μmol/L CP466722, or 10 μmol/L KU55933 to reach the experimental start point (i.e., 0 h). After preincubation, cells were exposed to mock-IR (Control) or IR (2 Gy) at the indicated time points (0–8 h). After irradiation, cells were incubated at 37°C for 30 min before being harvested (the times displayed in the figure represent the time of IR exposure and do not include the 30-min recovery time). To determine whether these compounds displayed a limited half-life with respect to inhibition of ATM kinase activity, ATM-intermolecular autophosphorylation at Ser1981 and phosphorylation of Chk2 (Thr68) were determined by Western blotting analysis (representative of several repeat experiments). A, CP466722 potently inhibits ATM for at least 8 h in culture. HeLa cells were preincubated with DMSO, 6 μmol/L CP466722, or 10 μmol/L KU55933 to reach the experimental start point (i.e., 0 h). After preincubation, the compounds were either left on the cells (+DMSO, +CP466722, and +KU55933) or removed (-DMSO, -CP466722, and -KU55933) and fresh media were added. After wash off, the cells were exposed to mock-IR (Control) or IR (2 Gy) at the indicated times (0–4 h). Irradiated cells were incubated at 37°C for 30 min before being harvested (the times displayed in the figure represent the time of IR exposure and do not include the 30-min recovery time). To determine whether the inhibition of ATM kinase activity had been reversed by removal of the compounds, ATM intermolecular autophosphorylation at Ser1981 and phosphorylation of downstream ATM targets were determined by Western blotting analysis (representative of several repeat experiments).
Transient inhibition of ATM sensitizes cells to IR induced DNA damage. One characteristic feature of cells deficient in functional ATM is their increased sensitivity to IR-induced DNA damage. This has been shown genetically using A-T cells, which have permanently disrupted ATM function or by chemical inhibition (KU5933), where ATM function has been disrupted for prolonged periods of time in cells (24). Based on the results indicating that inhibition of ATM kinase activity by these compounds was rapidly reversible, we were interested in whether transient inhibition of ATM could sensitize cells to IR. After pretreatment of HeLa cells with either DMSO, CP466722, or KU5933, the cells were exposed to the indicated doses of IR and allowed to recover for a period of 4 hours in the presence of DMSO or the inhibitors. The cells were then replated and incubated for a period of 10 days to allow for colony formation in the absence of inhibitors. Similar plating efficiencies were achieved in the presence (41 ± 3.7% and 39 ± 1.7%) or absence (42 ± 4.2% and 40 ± 2.0%) of CP466722 and KU5933, respectively, suggesting that neither compound affected cell plating nor cell viability. Transient exposure to either CP466722 or KU5933 sensitized cells to IR (Fig. 6). Because the compounds were only present for a 4-hour period and because the ATM pathway is reactivated rapidly upon removal of these compounds (Fig. 5B), it seems that a transient (4 hours or less) inhibition of ATM is sufficient to enhance the sensitivity of HeLa cells to IR. Importantly, no differences in clonogenic survival of cells from A-T patients were noted in the presence or absence of CP466722 (Supplementary Fig. S5), demonstrating that the radiosensitization caused by this compound was in fact due to ATM inhibition and not any off-target effects.

Discussion

Mammalian cells are constantly at risk from potentially lethal or mutagenic genomic lesions from both endogenous (e.g., free oxygen radicals) and exogenous (e.g., UV, IR) sources. As a result, eukaryotic cells have developed an intricate network of signal transduction pathways (DDR pathways) that allow them to sense and repair damaged DNA (1). Loss of function of critical proteins from these pathways can leave cells with enhanced sensitivity to DNA damaging agents (47). The ATM kinase is an important component of these DDR pathways, and cells deficient for ATM (A-T) display hypersensitivity to certain DNA damaging agents (3). Based on these observations, it has been proposed that specific inhibition of ATM function in combination with current radiotherapeutic/chemotherapeutic treatments may result in enhanced cancer cell killing (20, 22–24). This principal has been shown by the ability of specific antisense/small interfering RNA to attenuate ATM function and sensitize certain cancer cell lines to IR (48–50). Furthermore, the recent identification and characterization of the ATM inhibitor KU5933 has strengthened this hypothesis and shown that specific small molecule inhibition of ATM in vitro is capable of sensitizing human cancer cell lines to IR and topoisomerase poisons (24). Our aim in this study was to identify and characterize a novel inhibitor of the ATM protein kinase with a future goal of modifying this small molecule for characterization and use with in vivo models. In this paper, we identified the nontoxic compound CP466722 as an inhibitor of ATM and offer a comparison to the established ATM inhibitor KU5933 (24).

In response to IR, ATM initiates a signaling cascade and phosphorylates downstream targets (e.g., p53, Chk2, and SMC1) on characteristic sites which can be used as a measure of cellular ATM kinase activity (8, 10, 11, 13). CP466722 disrupts these cellular phosphorylation events in a dose-dependent manner in several different cell types and recapitulates the signaling defects observed in A-T cells (3). Closely related kinases (DNA-PK and ATR) share some downstream targets with ATM and phosphorylate common substrates (29, 35, 37); however, we found that CP466722 does not inhibit ATR kinase activity in vitro or the kinase activities of ATR or DNA-PK in cells. Furthermore, unlike the pan-Pi3K inhibitor wortmannin, CP466722 does not inhibit PI3K activity in cells. Interestingly, phosphorylation of Akt at Ser(173) is reported to be regulated by several PIKK family members, including DNA-PK, ATM, and mTOR (38). Although, Akt (Ser(173)) phosphorylation was inhibited by wortmannin, neither CP466722 nor KU5933 affected this modification. This implies that ATM

Figure 6. Transient inhibition of ATM kinase activity is able to sensitize cells to IR-induced DNA damage. HeLa cells were plated in triplicate and incubated for 24 h. Cells were preincubated with DMSO, 6 μmol/L CP466722, or 10 μmol/L KU5933 before being exposed to a range of doses of IR (0–10 Gy). Cells were incubated for 4 h after irradiation before being replated in fresh media in the absence of drug and incubated for a period of 10 d to allow for colony formation. To determine the effect of transient ATM inhibition by CP466722 and KU5933 in response to IR, the surviving colonies were counted and the data were presented on a log axis (represents the mean of three independent experiments ± SE).
is not required for this phosphorylation event under these experimental conditions and could indicate that these inhibitors do not affect additional PI3K-like protein kinases, such as mTOR (38). Similar to Ku55933, these results highlight CP466722 as a relatively specific inhibitor of ATM and a marked improvement on previous compounds used to inhibit ATM, such as wortmannin and caffeine. Extended analysis of CP466722 indicated that Abl and Src kinase activity were inhibited in vitro. However, BCR-Abl kinase activity was not affected in cells treated with this compound at doses that inhibit ATM, suggesting Abl is not a cellular target of CP466722. In contrast, autophosphorylation of Src (Tyr1141) was reduced by both CP466722 and Ku55933, although it is not clear whether these effects are direct or due to inhibition of signal transduction pathways that lead to Src kinase activation. This shows that there is still a need to modify and improve the specificity of these ATM inhibitors, and further characterization is required to identify and understand any potential off-target effects. It is noted that the lack of radiosensitization of A-T cells by CP466722 suggests that the inhibition of Src is not contributing to the radiosensitization induced by the drug.

Inhibition of ATM activity with CP466722 induced cellular effects indistinguishable from those seen in cells lacking ATM, including cell cycle checkpoint defects and radiosensitization (3). Similar to Ku55933, CP466722 rapidly and potently inhibits ATM over a period of several hours demonstrating reasonable stability in tissue culture (24). However, upon removal of either CP466722 or Ku55933 from tissue culture media, ATM kinase activity and the subsequent phosphorylation of downstream targets could be completely and rapidly restored. This ability to transiently inhibit ATM function followed by reactivation within such a short time frame is novel and opens new avenues for study of the ATM pathway. In effect, these inhibitors can be used as molecular switches to influence the immediate ATM-dependent DNA damage response and the subsequent repair process that contribute to cell survival.

Transient small molecule inhibition of ATM in vitro recapitulates the cellular A-T phenotype of increased sensitivity to IR while causing no additional sensitivity in an A-T cell line. However, the sensitization induced by these short-term exposures do not completely reflect the characteristic low-dose hypersensitivity phenotype of A-T cells, which could highlight a difference between long-term and short-term inhibition. In the study by Hickson and colleagues (24), long-term small molecule inhibition of ATM shows enhanced sensitivity to IR at low doses (1–2 Gy). Taken together, these results suggest that during and, for a short period of time, after IR, ATM plays an essential role in ensuring cellular survival that is not compensated for by other DDR pathways and cannot be rescued by reactivation of ATM. This concept is consistent with the proposed critical role of ATM activation and activity in the earliest steps of DSB repair (7). Further characterization of this observation with these inhibitors is still required to understand the role of ATM at these early time points. It could be informative to investigate the effects of transient inhibition and reactivation of ATM in future studies and determine how these influences cellular responses to DNA breakage, including which damage response proteins are recruited to DSBs and the kinetics of repair (7).

Because CP466722 can inhibit the ATM-signal transduction pathway in murine cells, it may be possible to use mouse models to begin to explore the effects of this compound in vivo. The observation that transient inhibition of ATM in tissue culture causes measurable hypersensitivity to IR could imply that stable and prolonged inhibition of ATM may not be needed to provide a therapeutic window. This concept requires further investigation and will require careful studies on drug delivery, distribution, stability, and activity in vivo.

In summary, we have identified and characterized a new inhibitor of ATM, which can be used to further characterize the function of the ATM-signaling pathway and the immediate molecular response to IR. In addition, this compound provides us with a novel chemical structure that can be modified to enhance potency, specificity, and ensure that second generation compounds can be taken forward into in vivo models. Further characterization of these inhibitors will help us to understand whether disruption of ATM function in vivo is a plausible approach for enhancing therapeutic potential.

Disclosure of Potential Conflicts of Interest

M. Kastan: Commercial research grant, Pfizer. The other authors disclosed no potential conflicts of interest.

Acknowledgments

Received 2/28/2008; revised 6/20/2008; accepted 7/11/2008.

Grant support: Pfizer, NIH grants CA1387, CA9632, and CA21765, and American Lebanese Syrian Associated Charities of St. Jude Children’s Research Hospital.

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We thank Dr. Richard Williams for providing advice and cells for this study. Dr. Christopher Bakkenist for his input with this research, all members of the Kastan laboratory for insightful suggestions throughout the course of this work, and Mukta Bagul, Yan Zhang, and Paul Bauer (Pfizer’s Research and Technology Center) for their contributions to this project.

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