Preclinical Evaluation of a Novel ATM Inhibitor, KU59403, In Vitro and In Vivo in p53 Functional and Dysfunctional Models of Human Cancer

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Abstract

Ataxia telangiectasia mutated (ATM) kinase signals DNA double-strand breaks (DSB) to cell-cycle arrest via p53 and DNA repair. ATM-defective cells are sensitive to DSB-inducing agents, making ATM an attractive target for anticancer chemo- and radiosensitization. KU59403 is an ATM inhibitor with the potency, selectivity, and solubility for advanced preclinical evaluation. KU59403 was not cytotoxic to human cancer cell lines (SW620, LoVo, HCT116, and MDA-MB-231) per se but significantly increased the cytotoxicity of topoisomerase I and II poisons: camptothecin, etoposide, and doxorubicin. Chemo- and radiosensitization by ATM inhibition was not p53-dependent. Following administration to mice, KU59403 distributed to tissues and concentrations exceeding those required for in vitro activity were maintained for at least 4 hours in tumor xenografts. KU59403 significantly enhanced the antitumor activity of topoisomerase poisons in mice bearing human colon cancer xenografts (SW620 and HCT116) at doses that were nontoxic alone and well-tolerated in combination. Chemosensitization was both dose- and schedule-dependent. KU59403 represents a major advance in ATM inhibitor development, being the first compound to show good tissue distribution and significant chemosensitization in in vivo models of human cancer, without major toxicity. KU59403 provides the first proof-of-principle preclinical data to support the future clinical development of ATM inhibitors.

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Introduction

DNA is constantly being damaged either from endogenous sources or environmental mutagens and carcinogens. DNA double-strand breaks (DSB) are particularly cytotoxic and cells mount a coordinated response of cell-cycle arrest and DNA repair in response to these lesions (1). The Ataxia Telangiectasia Mutated (ATM) kinase is a major coordinator of the DSB response and is the product of the ATM gene, which is defective in the disease Ataxia Telangiectasia (A-T) that is characterized by neurodegeneration, immunodeficiency, cancer predisposition, and an extreme hypersensitivity to ionizing radiation (IR) and other DSB-inducing agents (2). In response to DSBs, ATM initiates a cascade of phosphorylation events to induce cell-cycle arrest via p53 and other checkpoint proteins (reviewed in ref. 3) and promote DNA repair by both homologous recombination and nonhomologous end-joining (4, 5).

IR and topoisomerase poisons are important anticancer agents that induce DNA DSBs. It is estimated that 1 Gy of irradiation induces 1,000 single-strand breaks and 25 to 40 double-strand DNA breaks per diploid cell (6). Topoisomerase II poisons, by stabilizing the topoisomerase II–DNA cleavable complex, cause persistent protein-associated DNA DSBs, whereas topoisomerase I poisons stabilize the topoisomerase I–DNA cleavable complex to cause persistent single-strand breaks that are converted to DSB at replication. A-T cells display defective p53 induction and loss of cell-cycle arrest; however, lack of ATM also confers radiosensitivity in some p53-null mouse tissues suggesting the existence of a p53-independent ATM effector pathway (7). ATM inhibition is therefore an attractive approach to anticancer chemo- and radiosensitization (8) with potential benefits in both p53 functional and dysfunctional cancers.

The C-terminal domain of ATM contains the serine threonine kinase signature motif characteristic of the phosphoinositide 3-kinase (PI3K) family (9). The PI3K inhibitor LY294002 (Table 1) inhibits other members of the PI3K family (10), and we previously used scaffold hopping from LY294002 to develop KU5933 as a selective

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inhibitor of ATM (Table 1) that enhanced the cytotoxicity of IR and topoisomerase II poisons in human tumor cell lines (11). Further development identified KU600019 as a more potent and selective ATM inhibitor that radiosensitized glioma cells (12). However, neither compound has been evaluated in vivo. In the work presented here, we probe the p53 dependency of this class of compound and describe the novel ATM inhibitor KU59403 (Table 1), with increased potency and specificity against ATM, improved pharmacologic properties, and promising activity in animal models of human cancer.

Materials and Methods

Chemicals

The specific ATM inhibitors, KU55933 and KU59403, were kindly provided by KuDOS. Etoposide phosphate and irinotecan (CPT-11) were purchased from the National Health Service (UK). All other chemicals were purchased from Sigma unless stated otherwise. KU55933, KU59403, doxorubicin, camptothecin, and etoposide were dissolved in dimethyl sulfoxide (DMSO) for in vitro evaluation as 10 mmol/L stocks and stored at −20°C. All drugs were added to cells such that the final concentration of DMSO in culture media was 0.5% (v/v), and results were compared with controls incubated with 0.5% DMSO in media alone. Irinotecan (CPT-11, clinical grade, formulated in saline) and etoposide phosphate (etopophos, clinical grade, formulated in saline) were used in place of camptothecin and etoposide, respectively, for in vivo studies. KU59403 was administered at 10 mg/kg (the maximum administrable dose due to limited solubility) formulated in equimolar phosphoric acid, 5% (v/v) DMSO, 10% (w/v) encapsin, pH 4, and KU59403, formulated in equimolar phosphoric acid (Analar) in physiologic saline pH 4. All drugs for in vivo evaluation were formulated on the day of the experiment.

Enzyme inhibition

The activity of KU59403 against ATM and other PI3K family members isolated from HeLa cells was determined as previously described (11).

Cell lines and culture

LoVo, HCT116, and SW620 (human colon cancer); U2OS (human osteosarcoma); and MDA-MB-231 (human breast cancer) cells were purchased from the American Type Culture Collection. They were maintained at less than 30 passages from receipt using separate reagents for each cell line. HCT116 N7 cells (HCT-116 cells stably transfected with a plasmid containing HPV16 E6 cDNA such that p53 protein is degraded through the ubiquitin-proteasome pathway; ref. 13) were a gift from M. D’Incalci (Mario Negri Institute, Milan, Italy). U2OS p53ΔN expressing the p53-R248W dominant-negative mutant p53 were prepared by transfection of U2OS:PG13-Luc cells (14), and the failure to mount a p53 response to IR was confirmed in these cells (Supplementary Fig. S1). All cells were cultured in RPMI-1640 media supplemented with 10% (v/v) FBS, penicillin (50 units/mL), and streptomycin (50 units/mL) at 37°C in an atmosphere of 5% CO2 in air. Cells were

Table 1. Chemical structures and inhibition of ATM and related kinase activity by LY294002, KU55933, and KU59403

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical structure</th>
<th>ATN IC50, μmol/L</th>
<th>DNA-PK IC50, μmol/L</th>
<th>PI3K IC50, μmol/L</th>
<th>ATR IC50, μmol/L</th>
<th>PI4K IC50, μmol/L</th>
<th>mTOR IC50, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY294002a</td>
<td><img src="image1.png" alt="Chemical Structure" /></td>
<td>&gt;100</td>
<td>1.5</td>
<td>2</td>
<td>100</td>
<td>NDa</td>
<td>3</td>
</tr>
<tr>
<td>KU55933a</td>
<td><img src="image2.png" alt="Chemical Structure" /></td>
<td>0.013</td>
<td>2.5</td>
<td>1.7</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>9.3</td>
</tr>
<tr>
<td>KU59403</td>
<td><img src="image3.png" alt="Chemical Structure" /></td>
<td>0.003</td>
<td>9.1</td>
<td>10</td>
<td>&gt;100</td>
<td>&gt;10</td>
<td>14</td>
</tr>
</tbody>
</table>

NOTE: IC50 (μmol/L) against purified enzyme was calculated from sigmoid plots of increasing concentrations of inhibitor versus activity for each of the kinases. Data represent the mean of 5 independent experiments.

aData previously reported in ref. 11.
bPI4K value was not determined (ND) for LY294002 in these experiments.
confirmed to be free of mycoplasma contamination and LoVo, SW620, HCT116, and MDA-MB-231 were authenticated by short tandem repeat (STR) profiling (LGC Standards). The population doubling time of the cells was approximately 24 hours.

Cytotoxicity and growth inhibition studies

We determined the effect of KU55933 and KU59403 on cellular survival following exposure to X-ray irradiation or the topoisomerase II poisons, etoposide and doxorubicin, and the topoisomerase I poison, camptothecin (Supplementary Fig. S2) by clonogenic assay as described previously (15). Briefly, exponentially growing cells were exposed to the cytotoxic agent with or without KU55933 (10 μmol/L) or KU59403 (1.0 μmol/L) for 16 hours, and survival was calculated by comparison to the appropriate control (0.5% DMSO or ATM inhibitor alone). The dose modification ratio was calculated as the percentage surviving cells (compared with control) treated with the cytotoxic agent alone, divided by the percentage surviving cells treated with the cytotoxic agent and the ATM inhibitor.

KU55933 and KU59403 pharmacokinetic and tissue distribution studies

All in vivo experiments were reviewed and approved by the relevant institutional animal welfare committees and carried out according to national law and published guidelines (16). SW620 colorectal tumor cells (1 × 10^7 cells in 50 μL culture medium per animal) were injected subcutaneously into the flanks of female athymic nude mice (CD1 nu/nu, Charles River), and tissue distribution studies were conducted when tumors had reached a size of approximately 650 mm^3. KU59403 was given at 25 mg/kg to non–tumor-bearing female Balb/C mice or 50 mg/kg to SW620 tumor-bearing female nude mice. For comparison, KU55933 was administered at 10 mg/kg, 50 mg/kg to SW620 tumor-bearing female nude mice. For the relevant institutional animal welfare committees and distribution studies, KU59403 was given at 25 mg/kg to non–tumor-bearing female Balb/C mice or 50 mg/kg to SW620 tumor-bearing female nude mice. For comparison, KU55933 was administered at 10 mg/kg, which was the maximum administrable dose due to the limited solubility of KU55933 (i.e., 1 mg/mL even with the addition of 5% (v/v) DMSO and 10% (w/v) encapsin), to SW620-bearing nude mice. All doses were given at a dosing volume of 10 mL/kg via the intraperitoneal (i.p.) or intravenous route. Mice were bled under terminal anesthesia via cardiac puncture and the plasma fraction was stored at −20°C. Tissues were snap-frozen in liquid nitrogen and stored at −80°C until homogenization in PBS (1:3 w/v), using a stirrer macerator homogenizer (Werke GmbH & Co.) immediately before assay.

HPLC analysis of ATM inhibitors in plasma and tissue homogenates

KU55933 and KU59403 were extracted from plasma and tissue homogenates (50 μL) and analyzed by high-performance liquid chromatography (HPLC) as described previously (15). Plasma samples were quantified using a standard curve, prepared in plasma that was linear over the range: 0.05–10 μg/mL (r^2 > 0.9) with duplicate QA standards (at 0.1, 1, and 10 μg/mL). Tissue concentrations were calculated using the method of addition (17) to account for the efficiency of recovery and compensate for intersample variation.

Antitumor efficacy studies

CD-1 nude mice were implanted with SW620 or HCT116-N7 human cancer cell lines at 1 × 10^7 cells per animal (n = 5 per group). Treatment began when tumors were palpable (~5 × 5 mm^2, 8–10 days postimplantation) with normal saline (control animals), KU59403 as indicated in the Results section, alone or in combination with etoposide phosphate or irinotecan (CPT-11). For combinations, the first daily dose of KU59403 was administered immediately before etoposide phosphate or irinotecan unless otherwise indicated. Tumor volume was calculated from 2-dimensional electronic caliper (Mitutoyo) measurements using the equation a^2 × b/2 where a is the smallest measurement and b the largest. Data are presented as the median relative tumor volume (RTV), where the tumor volume for each animal on the initial day of treatment (day 0) is assigned an RTV value of 1.

Statistical analysis

Data were analyzed using GraphPad Prism software (GraphPad Software, Inc.). For the in vitro studies, significant differences between the effect of cytotoxic agent alone and cytotoxic agent plus KU59403 were determined by the Student t test (parametric). For in vivo studies, significant differences between the time take to reach RTV were determined by Mann–Whitney test.

Results

In vitro activity of KU59403 and p53 independence of chemo- and radiosensitiziation

KU59403 is a novel ATM inhibitor developed from LY294002 (Table 1), which is more potent against ATM than the previous lead KU55933 (IC50 = 3 vs. 13 nmol/L) and has at least 1,000 times greater specificity for ATM over other members of the PI3K family tested.

In contrast to the concentrations of 10 μmol/L of KU55933 and 3 μmol/L of KU600019 needed to induce in vitro chemo- and radiosensitization (11, 12), KU59403 was an effective chemosensitizer at a concentration of 1 μmol/L. At this concentration, KU59403 inhibited ATM activity in SW620 cells by more than 50% and at the higher concentration of 10 μmol/L, KU59403 also substantially inhibited ATM activity (Supplementary Fig. S3). KU59403 alone was not significantly cytotoxic to LoVo or SW620 cells (88% ± 7% and 91% ± 6% survival, respectively) but it enhanced camptothecin cytotoxicity (Fig. 1A, Table 2) in both cell lines with greater enhancement being observed in the LoVo than in the SW620 cells (7-fold; P = 0.038 vs. 4-fold; P = 0.014 at 10 nmol/L camptothecin). KU59403 also significantly enhanced the cytotoxicity of fixed concentrations of etoposide (0.1 and 1 μmol/L) or doxorubicin (10 or 100 nmol/L) in these cell lines, with greater enhancement of etoposide in SW620 cells and of doxorubicin in LoVo cells (Table 2).
There was no consistent difference in the enhancement of cytotoxicity in LoVo cells (wild-type p53) compared with SW620 cells (mutant p53) but as these cells were derived from different tumors, they could harbor other genotypic or phenotypic differences that might mask the effect of p53 status. For this reason, we investigated whether chemotherapeutic and radiosensitivity was enhanced by ATM inhibition in a p53-dependent manner using paired cell lines with functional or dysfunctional p53 using KU55933 as a model compound to confirm the data with KU59403 in proof of principle studies. KU55933 (10 μmol/L) sensitized p53 functional and dysfunctional HCT116 and U2OS cells to camptothecin to a similar extent (4- to 5-fold, Fig. 1B and Supplementary Table S1). Radiosensitization by KU55933 was greater in HCT116 than in U20S cells but the p53 status did not

Figure 1. In vitro chemosensitization. A, sensitization of SW620 (left) and LoVo cells (right) to camptothecin by KU59403. Cells were exposed to varying concentrations of camptothecin alone (filled circles, solid line) or in the presence of 1 μmol/L KU59403 (open circles, dashed line) for 16 hours before seeding for colony formation. Data, normalized to DMSO or KU59403 alone control, are mean ± SD from 3 independent experiments. B, survival of wt (circles) and p53 dysfunctional (triangles) HCT116 (left) and U2OS cells (right) following exposure to increasing concentrations of camptothecin in the presence (open symbols, dashed lines) or absence of KU55933 (filled symbols, solid line). Data, normalized to DMSO or KU59403 alone, are mean ± SD from 3 independent experiments. C, survival of wt (circles) and p53 dysfunctional (triangles) HCT116 (left) and U2OS cells (right) to increasing doses of X-rays in the presence (open symbols, dashed lines) or absence of KU55933 (filled symbols, solid line). Data, normalized to DMSO or KU59403 alone, are mean ± SD from 3 independent experiments. D, sensitization of SW620 HCT116, HCT116-N7, and MDA-MB-231 cells to etoposide by KU59403. Data are 1 μmol/L etoposide alone (white bars) or 1 μmol/L etoposide plus 1 μmol/L KU59403 (black bars) mean ± SD from 3 independent experiments.
Table 2. Cytotoxicity of etoposide, doxorubicin, and camptothecin, alone and in combination with KU59403 in LoVo and SW620 cells

<table>
<thead>
<tr>
<th>Cytotoxic drug</th>
<th>SW620</th>
<th>LoVo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotoxic alone</td>
<td>Enhancement factor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cytotoxic alone</td>
</tr>
<tr>
<td>Etoposide, 100 nmol/L</td>
<td>89 ± 8.3</td>
<td>35 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Etoposide, 1 μmol/L</td>
<td>3.8 ± 1.1</td>
<td>0.41 ± 0.23&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Doxorubicin, 10 nmol/L</td>
<td>50 ± 9</td>
<td>17 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Doxorubicin, 100 nmol/L</td>
<td>0.02 ± 0.01</td>
<td>0.01 ± 0.001</td>
</tr>
<tr>
<td>Camptothecin, 10 nmol/L</td>
<td>2.4 ± 0.2</td>
<td>0.71 ± 0.35&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NOTE: Cells were exposed to etoposide or doxorubicin, at the concentrations indicated, alone or in combination with 1 μmol/L KU59403 in a final concentration of 0.5% (v/v) DMSO for 16 hours. Data, normalized in comparison with DMSO or KU59403 alone control, as appropriate, are the mean ± SD of 3 independent experiments.

<sup>a</sup>Enhancement factor is defined as the survival with the cytotoxic alone/survival with cytotoxic + KU59403 in each individual experiment, and data are the mean ± SD of 3 independent experiments.

<sup>b</sup>Significant differences between cytotoxic drug alone versus cytotoxic + KU59403 is given by P < 0.01.

<sup>c</sup>Significant differences between cytotoxic drug alone versus cytotoxic + KU59403 is given by P < 0.05.

Affect radiosensitivity or enhancement by KU5933 (Fig. 1C and Supplementary Table S1). The p53 status of the cell did not have a consistent effect on chemosensitization of topoisomerase II poisons by KU5933 either, for example, p53 dysfunction conferred reduced sensitization to etoposide and doxorubicin in U2OS cells but had no significant impact in HCT116 cells (Supplementary Table S2). Consistent with the p53 independence of chemosensitivity, KU5933 increased the G<sub>2</sub> cell-cycle arrest induced by IR, camptothecin, doxorubicin, and etoposide to a similar extent in p53 functional and dysfunctional cells and did not affect DNA DSB formation or repair kinetics (Supplementary Figs. S4 and S5). These data were confirmed with KU9403 (1 μmol/L), which enhanced etoposide (1 μmol/L) cytotoxicity to a similar extent in HCT116 and HCT116-N7 cells by 2.3 ± 1.6-fold (P = 0.011) and 3.8 ± 2.5-fold (P = 0.019), respectively, and in the p53-mutant SW620 cells and human breast cancer cell line, MDA-MB-231, sensitization was 11.9 ± 4.7 (P < 0.0001) and 3.8 ± 1.8-fold (P = 0.006), respectively (Fig. 1D). Inhibition of IR-induced ATM activity by KU9403 (1 μmol/L) was approximately 50% in MDA-MB231 cells and more than 50% in HCT116 cells that have low ATM expression and approximately 50% in MDA-MB231, sensitization was 11.9 ± 4.7 (P < 0.0001) and 3.8 ± 1.8-fold (P = 0.006), respectively (Fig. 1D). Inhibition of IR-induced ATM activity by KU9403 (1 μmol/L) was approximately 50% in MDA-MB-231 cells and more than 50% in HCT116 cells that have low ATM expression and activity (Supplementary Fig. S3). These data indicate that p53 status has no major impact on sensitization by KU9403 and that SW620 cells, where a 12-fold enhancement was observed, are the most susceptible to etoposide sensitization by ATM inhibition. On the basis of these data, SW620 tumors treated with etoposide were chosen as our primary model system for the evaluation of KU9403 in <em>in vivo</em> studies.

**Pharmacokinetics**

As part of initial studies, plasma and tumor concentrations of drug were measured at 1 and 4 hours after administration of a single dose of KU9403 at 50 mg/kg i.p. and KU9593 at the maximum administrable dose of 10 mg/kg. The plasma concentration of KU9403 was 5 μmol/L and maintained for at least 4 hours. In comparison, plasma levels of KU5933 were just over 1 μmol/L, consistent with the 5-fold lower dose administered (Fig. 2A). KU9403 accumulated in tumor tissue up to the 4-hour time point with a concentration at this time of 1.9 μmol/L, which is greater than that shown to be necessary for activity in the <em>in vitro</em> studies (Fig. 2A). In contrast, the levels of KU5933 in the tumor were below the limit of detection (0.5 μmol/L). To determine the pharmacokinetics of KU9403 in normal tissues, the compound was administered to female Balb/C mice at 25 mg/kg intravenously (Fig. 2B). In contrast to the previous experiment, KU9403 was cleared rapidly from the plasma, and at 4 hours, the plasma concentration was less than 0.1 μmol/L. This difference could be due to different route of administration, different dose- or strain-specific metabolism. There was, nevertheless, substantial accumulation and retention in the tissues, especially the liver, indicating that hepatic clearance may be the main route of elimination of this compound. At this dose and route of administration, KU9403 achieved concentrations in tissues in excess of those required for <em>in vitro</em> chemosensitization.

**Antitumor efficacy studies**

To investigate whether the marked chemosensitization by KU9403 observed <em>in vitro</em> could be reproduced <em>in vivo</em>, we treated mice bearing SW620 tumor xenografts with etoposide phosphate (etopophos) at a fixed dose of 11.35 mg/kg (equivalent to 10 mg/kg free etoposide) i.p. daily for 5 days or irinotecan (2.5 mg/kg i.p.) daily for 5 days alone and in combination with KU9403. We also investigated the dose and schedule dependency of KU9403.
To investigate the enhancement of etopophos by KU59403 in a different xenograft model, mice bearing HCT116-N7 tumors were treated with vehicle alone, etopophos 11.35 mg/kg i.p. daily for 5 days, and/or KU59403 25 mg/kg i.p. twice daily for 5 days (Supplementary Fig. S4B). These tumors grew rapidly with tumors reaching RTV4 at 4.5 days, and HCT116-N7 tumors were resistant to etopophos alone (median time to RTV4 = 5.5 days). However, the etopophos-induced tumor growth delay was extended to 8.5 days by co-administration of KU59403, representing a 300% enhancement of etopophos activity that was statistically significant (Mann–Whitney test, \( P = 0.037 \)). Toxicity, as measured by body weight loss, was tolerable and transient (Supplementary Fig. S5B).

Irinotecan, a member of the camptothecin group of compounds, is commonly used in the treatment of colon cancer. In this study, irinotecan alone caused an initial modest regression of the SW620 tumor followed by rapid regrowth resulting in a tumor growth delay of 7.5 days, which was extended to 19.5 days by the co-administration of KU59403. This represents a 144% enhancement of irinotecan-induced tumor growth delay, which was significantly different from irinotecan alone (\( P = 0.032; \) Fig. 3C, Table 3). There were no unacceptable adverse effects on animal body weights at any of the doses given in this study (Supplementary Fig. S5C). As is clearly indicated from these data, enhancement of the efficacy of etopophos can be obtained in the SW620 and HCT116 xenograft models, and of irinotecan in the SW620 model, by combination with KU59403 with little enhancement of toxicity (measured by body weight loss).

Discussion

We had previously identified KU55933 as a potent and selective inhibitor of ATM (11), and subsequently KU600019 has been identified as a more potent ATM inhibitor (12). Fortunately, although these compounds provided in vitro evidence that inhibiting ATM induced chemosensitivity and radiosensitization in tumor cell lines, to date, there have been no in vivo investigations with small-molecule ATM inhibitors. Here, we described KU59403, a novel inhibitor of the ATM kinase that is more potent (IC50 = 3 nmol/L) and specific (at least 1,000-fold selective for ATM compared with the other members of the PIKK family tested) than previously described compounds of this class. As well as improved potency over KU55933, KU59403 also exhibits improved solubility, enabling us to determine the effect of ATM inhibition in animal models of human cancer for the first time.

KU59403 had no inherent cytotoxicity in vitro at a concentration (1 \( \mu \)mol/L) sufficient to cause marked chemopotentiation of topoisomerase I and II poisons, making it the most potent ATM inhibitor described to date.
Enhancement of etoposide cytotoxicity, ranging from 3- to 12-fold, was observed in a panel of human tumor cell lines with the greatest sensitization observed in SW620 cells. Interestingly, KU59403 only induced 2- to 3-fold sensitization of etoposide in HCT116 cells, which have been reported to have reduced ATM expression due to promoter methylation (18) and defects in MRE11 (19). Sensitization in MDA-MB-231 cells, which are reported to have mutated ATM (20) was also relatively modest. Both of these cell lines had reduced ATM activation by IR (~4-fold) in comparison to SW620 cells (6- to 7-fold). Studies in matched p53-proficient and -deficient cell lines showed that p53 status had no impact on chemosensitization by KU59403 or KU55933, and p53 status was not a determinant of the effect of KU55933 on cell-cycle arrest or DNA DSB repair. Cytotoxic drug or IR exposure resulted in G2 arrest in cells with both wild-type and dysfunctional p53 suggesting that G1 checkpoints were compromised in these cells irrespective of p53 status (21). The G2 arrest was enhanced by KU49403 independently of p53 status but whether this reflects further impairment of the G1 checkpoint, or that ATR signaling to the G2 checkpoint is increased when ATM is inhibited, remains to be determined.

Pharmacokinetic investigation of KU59403 revealed a more rapid clearance in Balb/C mice after an intravenous

Figure 3. Antitumor efficacy of KU59403 in combination with topoisomerase poisons. A, SW620 xenograft: KU59403 in combination with etoposide phosphate. CD-1 nude mice were treated with vehicle control (filled circles, solid line), KU59403 12.5 mg/kg 2x daily × 5 (open circles, dashed line), etoposide phosphate 10 mg/kg daily × 5 alone (filled triangles, solid line) or in combination with KUS4903 12.5 mg/kg 2x daily × 5 (open triangles, dashed line), KUS4903 6 mg/kg 2x daily × 5 (open inverted triangles, solid line), or KUS4903 25 mg/kg 2x daily × 5 (open squares, dashed line). Growth of SW620 xenografts is presented as the median RTV of groups of 5 animals. B, HCT116 N7 xenograft: KUS4903 in combination with etoposide phosphate. CD-1 nude mice were treated with vehicle control (filled circles, solid line), etopophos 10 mg/kg daily × 5 alone (filled triangles, solid line) or in combination with KUS4903 25 mg/kg 2x daily × 5 (open triangles, dashed line). Growth of HCT116-N7 xenografts is presented as the median RTV of groups of 5 animals. C, SW620 xenograft: KUS4903 in combination with irinotecan. CD-1 nude mice were treated with vehicle control (filled circles, solid line), KUS4903 12.5 mg/kg 2x daily × 5 (open circles, dashed line), irinotecan 2.5 mg/kg daily × 5 alone (filled triangles, solid line) or in combination with KUS4903 25 mg/kg 2x daily × 5 (open triangles, dashed line). Growth of SW620 xenografts is presented as the median RTV of groups of 5 animals.
dose but that plasma concentrations were maintained for at least 4 hours in tumor-bearing CD1 nude mice after intraperitoneal administration. Whether this difference in clearance reflects the route of administration, dose or strain effects were not determined. The pharmacokinetic studies in tumor-bearing mice indicated that levels of KU59403 sufficient for chemosensitization in vitro could be maintained in the tumor for at least 4 hours. Although levels of KU59403 in excess of those required for chemosensitization and radiosensitization in vitro were also detected in normal tissues for at least 4 hours following a dose of 25 mg/kg i.p., which could potentially have toxic consequences. KU59403 was nontoxic alone and did not cause a profound increase in either etoposide or irinotecan toxicity.

Similar to the in vitro studies, KU59403 alone had no impact on tumor growth rate. However, it did enhance the antitumor activity of etoposide against SW620 xenografts in a dose- and schedule-dependent manner. Significant sensitization was seen with a single daily dose of KU59403 at 12.5 mg/kg; administration but splitting the same total dose into 2 separate injections of 6 mg/kg was not as effective. Increasing the dose of KU59403 to 25 mg/kg given twice daily resulted in the greatest chemosensitization with a 3-fold increase in etoposide-induced tumor growth delay in both SW620 and HCT116-N7 xenografts, in the absence of a significantly increased toxicity. This is in contrast to the in vitro data where KU59403 enhanced etoposide cytotoxicity to a greater extent (3- to 12-fold) in SW620 cells than HCT116-N7 cells (2- to 4-fold) and suggests that in vitro data do not entirely predict in vivo results. It is possible that the tumor microenvironment may influence the efficacy of the combination as we have previously observed with chemosensitization studies (22).

Interestingly, it would seem that it is necessary to have KU59403 present at the time of etoposide dosing to have an effect, as delaying the administration of KU59403 by only 4 hours completely abolished chemosensitization. As ATM signaling is proposed to be an early response to DNA DSB, these data confirm the need to inhibit ATM while DNA DSB are being induced.

We have also shown that KU59403 can be used to enhance the sensitivity of human colon cancer cell lines to topoisomerase I poisons both in vitro and in vivo. KU59403 was shown to enhance the activity of camptothecin in both SW620 and LoVo cells in vitro (4- and 7-fold, respectively) and gave a 144% enhancement of irinotecan efficacy in a SW620 human xenograft model. These data as a whole are very encouraging and support the further development of this class of compound.

In summary, our studies have shown that ATM is valid target for the development of drugs designed to improve the activity of certain cytotoxic anticancer therapies. KU59403 is a potent and selective inhibitor of ATM, which is without intrinsic cytotoxicity but is a potent enhancer of topoisomerase I and II poison cytotoxicity in vitro. We have shown that KU59403 increases the efficacy of topoisomerase I and II poisons in vivo without intrinsic toxicity despite normal tissue exposure. These data provide further proof-of-principle evidence for the strategy of inhibiting ATM as a therapeutic maneuver for anticancer therapy.

### Disclosure of Potential Conflicts of Interest

N.J. Curtin and D.R. Newell have received research funding from KuDOS Pharmaceuticals Ltd and AstraZeneca, the purchasers of KuDOS. A. Lau, N.M.B. Martin, C. Richardson, and A. Slade are former employees of KuDOS Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

---

**Table 3.** Chemosensitization of antitumor activity determined in mice bearing SW620 xenografts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nadir % starting body weight</th>
<th>Time to RTV4</th>
<th>Delay, a, d</th>
<th>Enhancement (%) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>96.3</td>
<td>6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KU59403 12.5 mg/kg 2x daily × 5</td>
<td>100</td>
<td>7</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Etophos (equivalent to 10 mg/kg etoposide) daily × 5</td>
<td>98.6</td>
<td>10.5</td>
<td>4</td>
<td>38</td>
</tr>
<tr>
<td>Etophos + KU59403 6 mg/kg 2x daily × 5</td>
<td>96.9</td>
<td>12</td>
<td>5.5</td>
<td>113</td>
</tr>
<tr>
<td>Etophos + KU59403 12.5 mg/kg 2x daily × 5</td>
<td>94.9</td>
<td>15</td>
<td>8.5</td>
<td>190</td>
</tr>
<tr>
<td>Etophos + KU59403 25 mg/kg 2x daily × 5</td>
<td>93.3</td>
<td>18</td>
<td>11.5</td>
<td>0</td>
</tr>
<tr>
<td>Etophos + KU59403 12.5 mg/kg 1x daily × 5 concurrent</td>
<td>96.5</td>
<td>16.5</td>
<td>10</td>
<td>150</td>
</tr>
<tr>
<td>Etophos + KU59403 12.5 mg/kg 1x daily × 5 h post</td>
<td>94.3</td>
<td>10</td>
<td>3.5</td>
<td>0</td>
</tr>
<tr>
<td>Irinotecan 2.5 mg/kg daily × 5</td>
<td>93.6</td>
<td>14.5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>KU59403 25 mg/kg daily × 5</td>
<td>99.7</td>
<td>12</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>Irinotecan + KU59403 25 mg/kg daily × 5</td>
<td>100</td>
<td>26</td>
<td>19.5</td>
<td>144</td>
</tr>
</tbody>
</table>

aDelay (days) is the tumor growth delay, which is calculated as the time to median relative tumor volume 4 (median RTV4) following relevant treatment minus time to median RTV4 in control mice.
bEnhancement (%) is calculated as [100 × (delay combination/delay cytotoxic alone)] – 100.
cIndicates statistically significant enhancement (P < 0.05).
dIndicates marginally statistically significant enhancement (P < 0.1).
Preclinical Evaluation of ATM Inhibitor, KU59403

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References

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Michael A. Batey, Yan Zhao, Suzanne Kyle, et al.


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