

Improved ATM kinase inhibitor KU-60019 radiosensitizes glioma cells, compromises insulin, AKT and ERK prosurvival signaling, and inhibits migration and invasion

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Abstract

Ataxia telangiectasia (A-T) mutated (ATM) is critical for cell cycle checkpoints and DNA repair. Thus, specific small molecule inhibitors targeting ATM could perhaps be developed into efficient radiosensitizers. Recently, a specific inhibitor of the ATM kinase, KU-55933, was shown to radiosensitize human cancer cells. Herein, we report on an improved analogue of KU-55933 (KU-60019) with K_i and IC_{50} values half of those of KU-55933. KU-60019 is 10-fold more effective than KU-55933 at blocking radiation-induced phosphorylation of key ATM targets in human glioma cells. As expected, KU-60019 is a highly effective radiosensitizer of human glioma cells. A-T fibroblasts were not radiosensitized by KU-60019, strongly suggesting that the ATM kinase is specifically targeted. Furthermore, KU-60019 reduced basal S473 AKT phosphorylation, suggesting that the ATM

kinase might regulate a protein phosphatase acting on AKT. In line with this finding, the effect of KU-60019 on AKT phosphorylation was countered by low levels of okadaic acid, a phosphatase inhibitor, and A-T cells were impaired in S473 AKT phosphorylation in response to radiation and insulin and unresponsive to KU-60019. We also show that KU-60019 inhibits glioma cell migration and invasion *in vitro*, suggesting that glioma growth and motility might be controlled by ATM via AKT. Inhibitors of MEK and AKT did not further radiosensitize cells treated with KU-60019, supporting the idea that KU-60019 interferes with prosurvival signaling separate from its radiosensitizing properties. Altogether, KU-60019 inhibits the DNA damage response, reduces AKT phosphorylation and prosurvival signaling, inhibits migration and invasion, and effectively radiosensitizes human glioma cells. [Mol Cancer Ther 2009;8(10):2894–902]

Introduction

Malignant glioma and its most aggressive form, glioblastoma multiforme, are devastating and inevitably lethal cancers of the brain whose victims have a life expectancy of only 12 to 15 months after diagnosis (1). The standard treatment for glioma is surgery followed by chemoradiation (1). The cellular and molecular biology of glioma is complex and characterized by a highly invasive and aggressive phenotype, due in part to derailed growth factor-mediated signaling, making these cancers refractory to conventional treatments (2). Thus, there is an urgent need for novel and more effective therapies.

Individuals with the autosomal genetic disease ataxia telangiectasia (A-T) show impaired growth, immune deficiencies, cerebellar degeneration, telangiectasia of the eye, and premature aging (3). At the cellular level, A-T cells are extremely sensitive to ionizing radiation, have impaired G₁-S, intra-S, and G₂-M checkpoints, and show elevated levels of chromosomal instability (4). The protein mutated in A-T (ATM) and other members of the phosphatidylinositol-3'-kinase-related kinase (PIKK) family, including DNA-PKcs and A-T and RAD3-related (ATR), are critical for the cellular response to DNA damage. Serving both complementary and backup roles, the PIKKs control a coordinated defense by the cell at multiple levels, including cell cycle checkpoints, DNA double-strand break repair, and apoptosis (5, 6), collectively referred to as the DNA damage response (DDR; ref. 7). A critical function of ATM is to act as a protein kinase, phosphorylating an ever-increasing number of targets in response to ionizing radiation. These targets in turn cooperatively orchestrate a global cellular response (8). ATM is believed to regulate double-strand break repair directly or indirectly through cell

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cycle checkpoint control, and inhibition or absence of ATM increases radiosensitivity (7, 9). Thus, ATM is an attractive target for tumor radiosensitization.

In addition to delineating the DDR, recent work has established functional interactions between ATM and growth factor-mediated signaling (see ref. 10 for a recent review). Amplification or upregulation of growth factor receptor tyrosine kinases and loss of the PTEN phosphatase are common events in gliomas and are considered signs of poor prognosis (11). These changes lead to enhanced prosurvival signaling via PI3K/AKT and RAS/RAF/MEK/ERK, resulting in increased proliferation, metastasis, invasion, and radioresistance (7, 11). ATM is known to control insulin-mediated signaling (12–14), which in turn regulates AKT signaling. ATM is also reported to modulate radiation-induced AKT signaling, but the mechanism underlying this response is unclear (14). In addition, our own results have shown that MEK/ERK signaling is modulated by ATM (15). Very recently, several studies showed that DNA-PKcs directly phosphorylates AKT at S473 in response to DNA damage (16–18). Furthermore, ATM phosphorylates DNA-PKcs on T2609 and regulates its function (19), and DNA-PKcs seems to regulate ATM protein levels and activity (20). Collectively, these studies suggest that ATM and DNA-PKcs could perhaps coregulate DNA damage-induced prosurvival signaling via AKT. Thus, inhibition of ATM signaling may compromise prosurvival signaling in addition to inhibiting more established cell cycle checkpoints and double-strand break repair targets.

Herein, we report on the radiosensitizing properties of a novel ATM kinase-specific small molecule inhibitor, KU-60019, that is more effective than its predecessor. In addition to establishing its improved radiosensitizing properties we also begin to characterize an ATM-regulated response that controls AKT phosphorylation, which in turn might compromise glioma migration and invasion. Thus, KU-60019 could perhaps be developed into a highly effective cancer drug that not only would work as a radiosensitizer but would also curtail tumor dispersal.

Materials and Methods

Chemical Synthesis and Structure of KU-60019

The chemical synthesis of 2-[(2R, 6S)-2, 6-dimethylmorpholin-4-yl]-N-[5-(6-morpholin-4-yl-4-oxo-4H-pyran-2-yl)-9H-thioxanthen-2-yl]-acetamide (KU-60019) is described in patent WO/2007/026157 (<http://www.wipo.int/portal/index.html.en>). The calculated molecular weight of KU-60019 (C₃₀H₃₂N₃O₅S) is 547.67.

Reagents

Anti-p(S15)-p53, -p(T68)-Chk2, -p(S473)-AKT, -AKT, -p(S136)-BAD, -BAD, -p(S9)-GSK3 β , and -p(S1981)-ATM antibodies and GST-GSK3 β substrate were purchased from Cell Signaling Technology, Inc. Anti- γ -H2AX (S139) antibody was from Millipore. Anti- β -actin, -p(T202/Y204)-ERK1/2, and -ERK2 antibodies were from Santa Cruz Biotechnology. Anti-GSK3 β , insulin, and okadaic acid were purchased

from Sigma Aldrich. Matrigel was purchased from BD Biosciences. SH-5 was purchased from EMD Biosciences, and PD184352 has been described (21). KU-60019, KU-55933, and KU-57788 were all from KuDOS Pharmaceuticals Ltd.

Cell Culture and Treatments

The U87 (22), U1242, and U1242/luc-GFP (23) human malignant glioma cells were cultured in α -MEM medium supplemented with 10% fetal bovine serum and antibiotics. Human primary GM02270 (normal fibroblasts) and GM05823 (A-T fibroblasts) cells (Coriell Institute for Medical Research) were cultured in MEM supplemented with vitamins, minimum-essential amino acids, nonessential amino acids (Invitrogen), and 15% fetal bovine serum. Glioma cells were originally obtained from the American Type Culture Collection and Dr. Allan Yates, Ohio State University, Columbus, OH, respectively. Routine characterization included the ability to form intracranial tumors in nude mice, and quantitative reverse transcriptase-PCR (U1242) expression profiling. The cells were not tested and authenticated by an external service provider. Irradiations were done using an MDS Nordion Gammacell 40 research irradiator with a 137-Cs source delivering a dose rate of 1.05 Gy/min.

Western Blotting

Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (BioRad) for Western blotting as previously described (15).

Cell Growth

Cell growth was determined by AlamarBlue (24). U1242 cells were serially diluted, allowed to attach for 6 h, and then exposed to KU-60019 at 3 μ mol/L. At days 1, 3, and 5 after seeding, AlamarBlue (AbD Serotec) was added to the medium to the recommended final concentration. Plates were incubated for 1 h at 37°C, fluorescence was determined on a Fluoro-Count plate reader (Packard; excitation 530 nm, emission 590 nm), and values were taken as a measure of cell growth.

Cell Survival

Trypan Blue/Fluorescence Activated Cell Sorting (FACS) Assay. Surviving fractions were calculated by determining the number of live cells in each sample relative to the untreated control sample after trypan blue and flow cytometry (described in more detail in the supplementary methods).

Clonogenic Survival. Clonogenic radiosurvival experiments were carried out as described (22, 25) in more detail in the supplementary methods.

Migration and Invasion Assays

Migration and invasion assays were carried out as described (26), and are outlined in more detail in the supplementary methods section.

Statistics

Linear regression, polynomial regression, and unpaired one- or two-tailed *t* tests were done as appropriate on triplicate (or more) data sets using GraphPad Prism 3.0 (GraphPad Software, Inc). *P* values are indicated as: *, < 0.05; **, < 0.01; and ***, < 0.001.

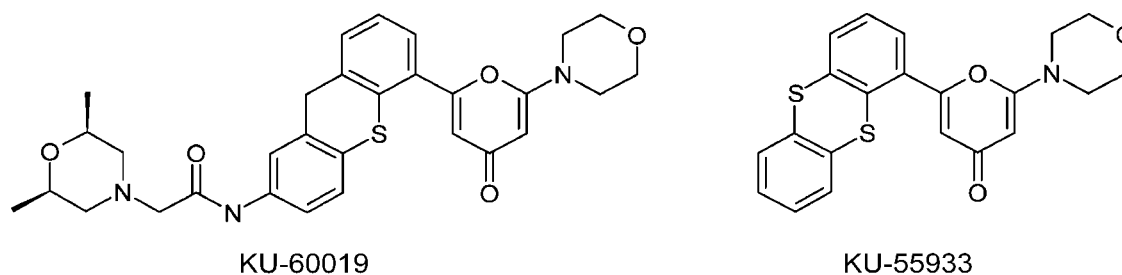


Figure 1. Chemical structures of KU-60019 and KU-55933.

Results

KU-60019 Is an Improved ATM Kinase-Specific Inhibitor

Recently, the novel and specific inhibitor of the ATM kinase, KU-55933, was identified in a screen of a small molecule library based on the relatively nonspecific phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (25). KU-55933 has an IC_{50} of 13 nmol/L and K_i of 2.2 nmol/L *in vitro* and is highly specific for the ATM kinase using a panel of 60 protein kinases (25). To improve the pharmacokinetics and bioavailability, a new more water-soluble analogue was synthesized that shares many if not all of the KU-55933 structural, pharmacologic, and biological effects (see patent WO/2007/026157). KU-60019 is an improved inhibitor of the ATM kinase with an IC_{50} of 6.3 nmol/L, approximately half that of KU-55933. The IC_{50} values for DNA-PKcs and ATR are 1.7 and >10 μ mol/L, respectively, almost 270- and 1,600-fold higher than that for ATM (data not shown). KU-60019 has similar if not identical target specificity as KU-55933, with little to no nonspecific target effects at 1 μ mol/L against a panel of 229 protein kinases (Supplementary Table S1) with PI3K (p110 β /p85 α), PI3K (p120 γ), and PI3K (p110 δ /p85 α) inhibited 9%, 3%, and 27% (data not shown), respectively (Millipore KinaseProfiler and PI3-Kinase HTRF assay). Notably, mTOR and mTOR/FKBP12 were not inhibited. The chemical structures of KU-60019 and KU-55933 are shown in Fig. 1.

KU-60019 Is a More Potent Inhibitor of the ATM Kinase than Is KU-55933

To begin determining the relative potency of KU-60019 and KU-55933 to block the ATM kinase in human glioma cells, we assessed the impact on ionizing radiation-induced phosphorylation of key ATM targets. ATM phosphorylates numerous proteins at specific positions, including p53 at S15, H2AX at S139 (γ -H2AX), and CHK2 at T68 (7, 8). In human U87 glioma cells, KU-55933 completely inhibited phosphorylation of p53 (S15) at 10 μ mol/L but not at 3 μ mol/L (Fig. 2A; compare lanes 4 to 6 with 8 and 9), whereas γ -H2AX levels were only partly reduced with 10 μ mol/L 1 hour after irradiation. By comparison, 3 μ mol/L KU-60019 completely inhibited p53 phosphorylation and partially inhibited at 1 μ mol/L (Fig. 2A; compare lanes 8 and 9 with 13 to 15). As with KU-55933, little to no effect on H2AX phosphorylation was seen 1 hour after irradiation. Because ATM is believed to phosphorylate H2AX at

S139 immediately after irradiation, with DNA-PKcs serving as backup (27, 28), we examined these responses at both 15 and 60 minutes after radiation (Fig. 2B). To determine the contribution of DNA-PKcs, we utilized the DNA-PKcs-specific inhibitor KU-57788 (NU7441; ref. 29). As before, KU-60019 at 3 μ mol/L completely inhibited p53 phosphorylation 15 minutes post-ionizing radiation, whereas inhibiting DNA-PKcs with KU-57788 (2.5 μ mol/L) did

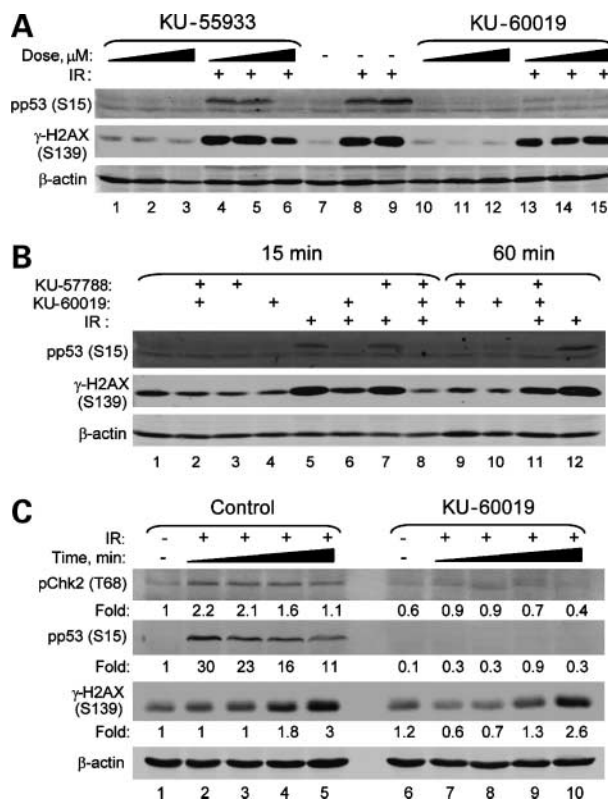


Figure 2. KU-60019 is a more effective inhibitor of the ATM kinase than is KU-55933. **A**, U87 cells were treated with KU-55933 or KU-60019 (0, 1, 3, or 10 μ mol/L) for 1 h, exposed to 10 Gy of ionizing radiation and collected for Western blot analysis after 1 h. **B**, U87 cells were treated with KU-57788 (2.5 μ mol/L), KU-60019 (3 μ mol/L), or both, exposed to 10 Gy of ionizing radiation, and collected for Western blot analysis after 15 or 60 min. **C**, U1242 cells were treated with KU-60019 (3 μ mol/L), exposed to 5 Gy, and collected for Western blot analysis after 5, 15, 30, or 60 min. Fold depicts phospho-protein levels normalized to β -actin levels. Drugs remained in the medium throughout the experiments.

not (Fig. 2B; compare lanes 5 to 7). Importantly, even 1 $\mu\text{mol/L}$ of KU-60019 almost completely blocked (>70%) p53 (S15) phosphorylation (Fig. 2B; compare lanes 8 and 9 with 13), suggesting that at the concentration used in the *in vitro* KinaseProfiler assay (Supplementary Table S1), KU-60019 almost completely inhibited the DDR in intact cells. As expected, $\gamma\text{-H2AX}$ levels were reduced significantly at 15 minutes with KU-60019 (Fig. 2B; compare lanes 5 and 6). In addition, when both KU-60019 and KU-57788 were added $\gamma\text{-H2AX}$ levels were reduced even further, close to levels detected in nonirradiated controls (Fig. 2B; compare lanes 6 to 8). However, at 60 minutes the combined inhibitory effect of KU-60019 and KU-57788 was reduced as indicated by the increased $\gamma\text{-H2AX}$ levels (compare lanes 8 and 11). These results suggest that ATM is the principal kinase of p53 (S15) and H2AX (S139) phosphorylation at early times after irradiation, with DNA-PKcs and ATR serving as complementary and backup kinases, respectively, in agreement with previous reports (27, 28).

The ATM-mediated radiation response was also examined in U1242 glioma cells. Contrary to U87 cells (p53 wild type, and deleted PTEN), U1242 cells express mutant p53 (H175R) and wild-type PTEN (30), and are highly invasive *in vivo* (23). In these cells, radiation-induced CHK2 phosphorylation (T68) was completely inhibited by KU-60019 at 3 $\mu\text{mol/L}$ up to 1 hour after irradiation (Fig. 2C). Moreover, just as with U87 cells, we found that p53 phosphorylation was completely inhibited and $\gamma\text{-H2AX}$ partially inhibited, especially at times ≤ 15 minutes. Altogether, KU-

60019 is 3- to 10-fold more potent than KU-55933 at blocking radiation-induced phosphorylation of key ATM protein targets in human glioma cells.

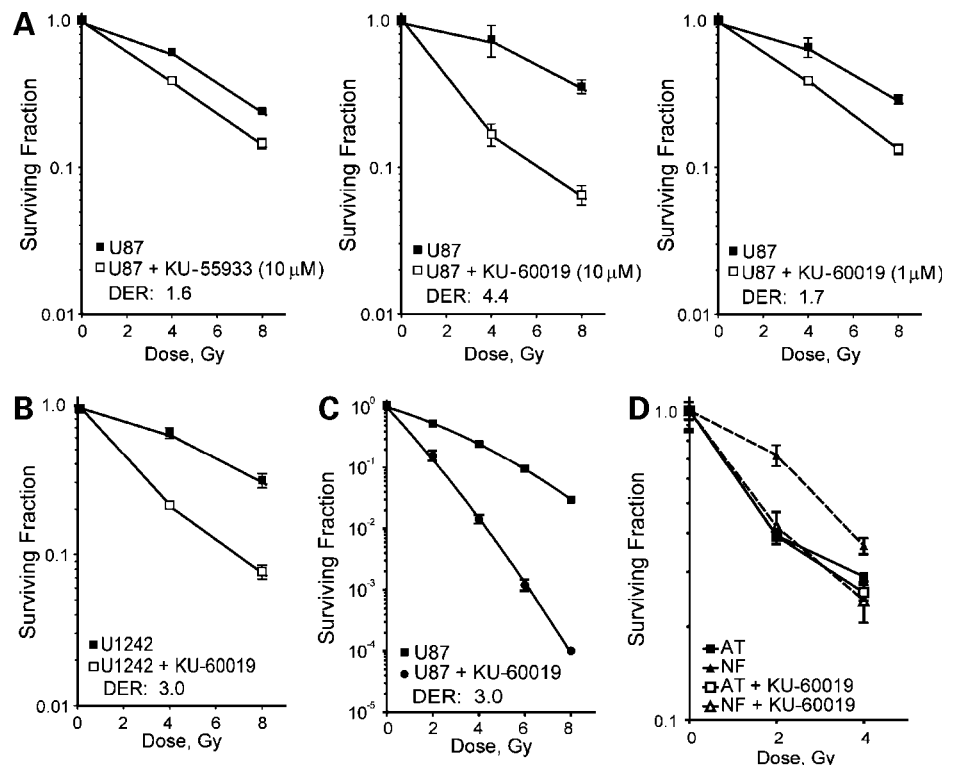
KU-60019 Is a More Potent Radiosensitizer than KU-55933

We then examined the relative potency of KU-60019 and KU-55933 at radiosensitizing human glioma cells. Using a novel assay we have developed combining the common trypan blue viability staining with flow cytometry, we found that KU-55933 and KU-60019 at 10 $\mu\text{mol/L}$ resulted in dose-enhancement ratios of 1.6 and 4.4, respectively (Fig. 3A, *left* and *middle*). A 10-fold lower concentration of KU-60019 (1 $\mu\text{mol/L}$) resulted in a dose-enhancement ratio of 1.7, which was similar to the radiosensitization seen with 10 $\mu\text{mol/L}$ of KU-55933 (Fig. 3A, *left*). Similarly, KU-60019 at 3 $\mu\text{mol/L}$ radiosensitized U1242 cells to a level between that of U87 cells treated with 1 and 10 $\mu\text{mol/L}$ of KU-60019 (Fig. 3B). To support these results, colony-forming radiosurvival experiments were done with U87 cells and normal fibroblasts (Fig. 3C and Supplementary Fig. S2B). As expected, we found that KU-60019 at 3 $\mu\text{mol/L}$ severely impaired radiosurvival, resulting in dose-enhancement ratios of 3.0 and 2.8, respectively. Thus, little to no tumor specificity of KU-60019 was noted, as expected. Together, these results show that KU-60019 is approximately 10 times more potent than KU-55933 at radiosensitizing human glioma cells.

KU-60019 Specifically Targets the ATM Kinase to Radiosensitize Cells

To show that KU-60019 is specific for ATM, we treated h-TERT-immortalized normal and A-T fibroblasts with

Figure 3. KU-60019 radiosensitizes U87 and U1242 human glioma cells and normal but not A-T fibroblasts. Cells were treated with KU-60019 or KU-55933 at the indicated concentrations for 1 h prior to ionizing radiation. Drugs were removed 16 h post-ionizing radiation. Surviving fractions were determined by trypan blue/FACS assay (A, B and D) or crystal violet staining and colony counting (C). **A**, U87 radiosurvival 7 d post-ionizing radiation \pm KU-55933 at 10 $\mu\text{mol/L}$ (*left*), \pm KU-60019 at 10 $\mu\text{mol/L}$ (*middle*), or \pm KU-60019 at 1 $\mu\text{mol/L}$ (*right*). **B**, U1242 radiosurvival 4 d post-ionizing radiation \pm KU-60019 at 3 $\mu\text{mol/L}$. **C**, U87 radiosurvival 14 d post-ionizing radiation \pm KU-60019 at 3 $\mu\text{mol/L}$. **D**, radiosurvival of normal (NF) or A-T fibroblast 7 d post-ionizing radiation \pm KU-60019 at 10 $\mu\text{mol/L}$. Data points, surviving cells plotted as fraction of control ($-$ ionizing radiation); error bars; SE; $n = 3$. Where error bars are not seen they are obscured by symbols.



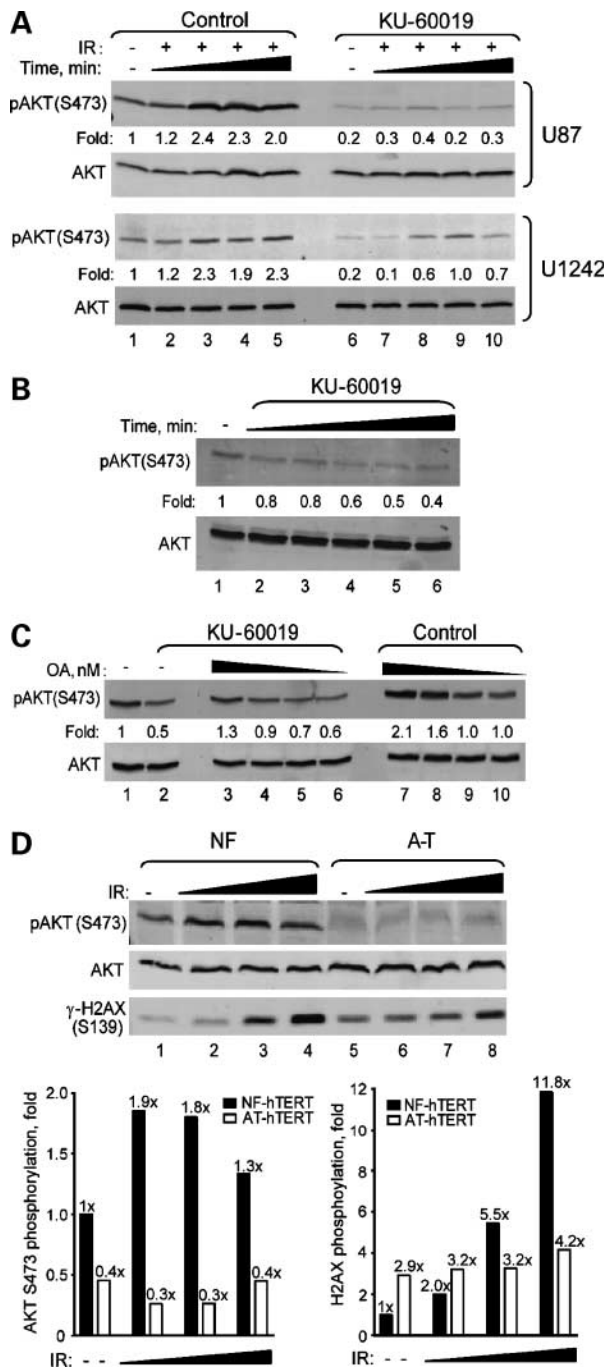


Figure 4. Radiation-induced and basal AKT (S473) phosphorylation is blocked by KU-60019 in human glioma cells and is impaired in A-T cells. **A**, U87 or U1242 cells were treated with KU-60019 at 3 μmol/L 1 h prior to irradiation with 5 Gy. Cells were collected at 5, 15, 30, or 60 min after irradiation and processed for Western blot analysis. **B**, U87 cells were treated with KU-60019 (3 μmol/L) and harvested for Western blot analysis after 5, 15, 30, 45, and 60 min. **C**, U87 cells were treated with or without KU-60019 (3 μmol/L) and decreasing concentrations of okadaic acid (100, 30, 10, 3 nmol/L) for 1 h, then harvested for Western blot analysis. Fold depicts phosphorylated AKT levels compared with untreated control levels (lane 1) normalized to total AKT protein levels. **D**, A-T and normal fibroblasts (h-TERT) were exposed to ionizing radiation (0, 2, 5, or 10 Gy) and collected for Western blot analysis after 15 min. Data points, relative phosphorylation levels.

KU-60019 prior to ionizing radiation and determined radiosurvival by trypan blue/FACS assay (Fig. 3D). As expected, only the normal fibroblasts were radiosensitized by KU-60019 and not the A-T fibroblasts. This result strongly suggests that KU-60019 is an ATM kinase-specific radiosensitizer.

KU-60019 Modulates Phosphorylation of AKT at S473

It was recently reported that ATM regulates the phosphorylation of AKT at S473 in response to insulin and ionizing radiation (14). However, this effect is likely indirect as the S473 site is not a consensus S/T-Q ATM kinase motif (31). DNA-PKcs has been shown to regulate AKT phosphorylation, but contrary to ATM, DNA-PKcs directly phosphorylates AKT at S473 in response to DNA damage (16, 17). We previously showed a role for ATM in regulating ERK pro-survival signaling in double-strand break repair (15), and ERK and AKT signaling are coregulated to some extent via RAS in response to ionizing radiation (see ref. 10 for a recent review). Therefore, we tested whether KU-60019 affects S473 AKT phosphorylation in human glioma cells (Fig. 4). After a dose of 5 Gy to U87 cells, phospho-AKT (S473) levels increased in a time-dependent manner and peaked at 2.4-fold after 15 minutes (Fig. 4A, lane 3). KU-60019 almost completely blocked this increase, and in fact, seemed to reduce phosphorylation below the level of an unirradiated control (Fig. 4A; compare lanes 1 and 6). Pooled data from several independent experiments showed that KU-60019 reduced basal AKT S473 phosphorylation by 70% (Supplementary Fig. S1A). Similar responses were seen with U1242 cells, i.e., AKT phosphorylation increased in response to radiation and KU-60019 almost completely abrogated basal and radiation-induced AKT S473 phosphorylation (Fig. 4A). Additionally, we noticed that KU-55933 attenuated radiation-induced AKT T308 phosphorylation (Supplementary Fig. S1B). A time course of S473 dephosphorylation with KU-60019 alone showed an effect as early as 5 minutes after the addition of drug (Fig. 4B). KU-60019 and KU-55933 also reduced the phosphorylation of (S136) BAD and (S9) GSK3β *in vivo* and *in vitro* (Supplementary Fig. S1C and D), suggesting that AKT activity is reduced.

These results show that KU-60019 blocks pro-survival signaling resulting in reduced AKT (S473) phosphorylation in several human glioma cell lines. This response occurs in both p53 wild-type (U87) and mutant (U1242) backgrounds, regardless of PTEN status, and seems independent of DNA damage because AKT phosphorylation levels were below those seen in untreated cells. However, the radiation-induced increase in S473 AKT phosphorylation was not completely inhibited, suggesting that phosphorylation (perhaps by DNA-PKcs) still occurred (Fig. 4A; compare lanes 6 to 10). Combined, the results suggest that a protein phosphatase is acting on phosphorylated AKT and that this phosphatase may be under the control of the ATM kinase.

To examine whether a phosphatase could be involved in the response to KU-60019, we treated cells with okadaic acid, a known inhibitor of PP1, PP2A, PP4-6 (32). In the presence of ≥30 nmol/L okadaic acid, the effect of

KU-60019 on S473 phosphorylation was reduced by >50%. In fact, okadaic acid by itself increased AKT S473 phosphorylation >2-fold compared with untreated control (Fig. 4C). These data suggest that an okadaic acid-sensitive phosphatase regulated by ATM modulates AKT phosphorylation, although we cannot at the present time rule out alternative mechanisms. Further studies will be necessary to identify this putative phosphatase.

Radiation- and Insulin-Induced AKT Phosphorylation Is Impaired in Human A-T Fibroblasts

To obtain further indication that AKT signaling is impaired when ATM is inactive, as our observations with KU-60019 suggest, we examined this response in A-T and normal fibroblasts immortalized with h-TERT. The normal fibroblasts showed low basal levels of phospho-(S15) p53, which increased after ionizing radiation (data not shown). On the other hand, A-T (h-TERT) cells did not, and also did not produce any radiation-induced S1981 ATM phosphorylation (Supplementary Fig. S2A). Normal fibroblast-hTERT cells are also radiosensitized by KU-60019 to a similar degree as our glioma lines, showing that KU-60019 is not tumor cell-specific (Supplementary Fig. S2B), as expected. A Western blot of extracts from untreated and irradiated A-T and normal fibroblasts showed that, indeed, S473 AKT phosphorylation increased in normal fibroblasts after radiation (1.9-fold after 2 Gy), whereas this was not seen with A-T cells (Fig. 4D). In addition, extracts from A-T cells showed basal AKT phosphorylation levels at least 2-fold lower than those from untreated normal fibroblasts, and radiation at any dose did not increase AKT phosphorylation (Fig. 4D). When γ -H2AX levels were examined, a dose-dependent increase (2- to ~12-fold) was observed with normal fibroblasts whereas the A-T cells only produced a slight increase after 10 Gy (Fig. 4D). Furthermore, a time-dependent increase in AKT phosphorylation was seen as early as 15 minutes in normal fibroblasts but not A-T cells (data not shown). All combined, A-T cells are severely impaired in basal and radiation-induced AKT signaling whereas normal fibroblasts are proficient, suggesting that ATM is important for regulating radiation-induced AKT S473 phosphorylation in human cells. Peak AKT phosphorylation occurs at a relatively low dose of radiation (2 Gy) at early times (≤ 15 minutes). These observations with A-T and normal fibroblasts support our findings with KU-60019 in glioma cells and implicate the ATM kinase in regulating AKT phosphorylation.

If KU-60019 activates a phosphatase that removes the phosphates on S473, and T308, it would be interesting to see whether stimulation of AKT phosphorylation by insulin would also be affected by KU-60019. Thus, U87 cells were treated with insulin with or without KU-60019 followed by p-AKT Western blotting. We noticed a ~50% reduction in AKT phosphorylation with KU-60019 present (Supplementary Fig. S3A), suggesting that the same ATM-dependent phosphatase acts on AKT regardless of whether stimulation is from radiation, insulin, or basal levels of growth. To see whether A-T (h-TERT) cells are also impaired in AKT phosphorylation in response to insulin, we stimulated normal fibroblast and A-T fibroblasts with insulin \pm KU-60019. We found that A-T cells are impaired in AKT phosphorylation

in response to insulin compared with normal fibroblasts (~60% less phosphorylation), in agreement with previous reports (12–14), and that KU-60019 reduced AKT phosphorylation in the normal fibroblasts (~45%) as it did in glioma cells. However, KU-60019 did not affect S473 phosphorylation in A-T cells in response to insulin, again, suggesting that KU-60019 is specific for the ATM kinase (Supplementary Fig. S3B). These results show that insulin signaling resulting in AKT phosphorylation is impaired in A-T cells and that KU-60019 reduces AKT phosphorylation in response to insulin in both glioma cells and normal fibroblasts. The lack of an effect of KU-60019 on insulin stimulation of AKT phosphorylation in A-T cells is in line with the inability of KU-60019 to radiosensitize A-T cells, and further supports the conclusion that KU-60019 is specific for the ATM kinase.

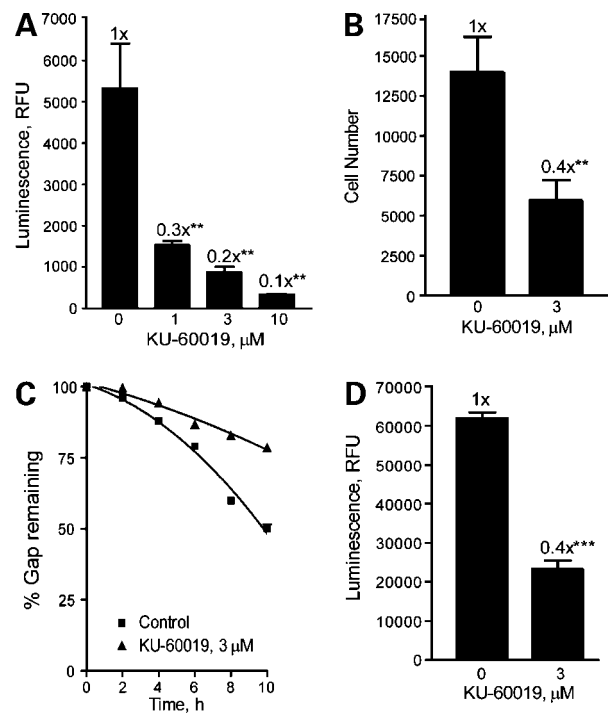


Figure 5. KU-60019 inhibits migration and invasion of human glioma U87 and U1242 cells *in vitro*. **A**, relative migration of U87-Luc cells through 8- μ m pore membranes in the presence of KU-60019 (0, 1, 3, or 10 μ mol/L). Quantification of cell numbers was done by determining luciferase activity 6 h postseeding. *Data points*, relative luminescence per well; *error bars*, SE; *n* = 4; RFU, relative fluorescence units. Fold (x) depicts relative migration compared with untreated control (-KU60019). **B**, relative invasion of U87 cells through Matrigel-coated inserts \pm KU-60019 at 3 μ mol/L. Cells were collected and counted 48 h after seeding. *Data points*, total number of cells/well; *error bars*, SE; *n* = 5. Fold (x) depicts relative invasion compared with untreated control. **C**, scratch assay of U1242 cells. Closure of the scratch was measured over time in the presence or absence of KU-60019 at 3 μ mol/L. *Data points*, mean width of scratch; *error bars*, SE; *n* = 3. Where error bars are not seen they are obscured by symbols. Rate of gap closure was 50% slower in the KU-60019 treated cells at 10 h ($P \leq 0.0005$). **D**, relative invasion of U1242 cells through Matrigel inserts in the presence or absence of KU-60019 at 3 μ mol/L. Cell numbers were determined by ATP luciferase assay after 48 h. *Data points*, RFU per well; *error bars*, SE; *n* = 3. Fold (x) depicts relative invasion compared with untreated control (-KU-60019). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant.

KU-60019 Inhibits Migration and Invasion of Human Glioma Cells *In vitro*

Because AKT and ERK signaling regulate glioma migration and invasion perhaps via deregulated receptor tyrosine kinase-mediated signaling (2), we wanted to determine whether KU-60019 affects these very critical aspects of glioma pathophysiology. Thus, we carried out a migration assay of U87 cells with or without KU-60019 using established *in vitro* conditions (Fig. 5). We found that KU-60019 inhibited migration of U87 cells $\geq 70\%$ in a dose-dependent manner (Fig. 5A). Furthermore, invasion is a hallmark of malignant gliomas (2). We found that invasion through Matrigel was inhibited $\sim 60\%$ by KU-60019 (Fig. 5B). The U1242 cells show a more invasive phenotype than U87 tumors when grown as orthotopic mouse xenografts (ref. 23; data not shown). Using an alternative *in vitro* test for migration/motility, the "scratch" or "wound healing" assay, we show that KU-60019 also significantly inhibited U1242 migration by at least 50% (Fig. 5C). In addition, KU-60019 inhibited invasion of U1242 cells by $\sim 60\%$ (Fig. 5D). In line with these findings, we found that KU-60019 at 3 $\mu\text{mol/L}$ suppressed the growth of U1242 cells by $\sim 40\%$ over a 5-day period (Supplementary Fig. S4). However, the reduction was only noted at later times. Altogether, these results show that KU-60019 inhibits migration/motility, invasion, and to some extent the growth of human glioma cells *in vitro*.

Inhibition of AKT or MEK/ERK Signaling Does Not Enhance KU-60019 Radiosensitization

Because AKT and MEK/ERK signaling regulate cell growth and both pathways are frequently upregulated in cancer cells, both have been explored as potential therapeutic targets with varying levels of success. Drugs inhibiting either the AKT or MEK kinases are cytostatic, and synergy with radiation has been inconsistent and seems to be cell type- and cell state-dependent (33–36). Thus, we wanted to determine whether inhibition of MEK/ERK or AKT signaling enhanced killing beyond inhibition of ATM alone. We reasoned that if KU-60019 indeed inhibits MEK/ERK and AKT signaling, as suggested here and in our previous study (15), then no further radiosensitization should be observed when KU-60019 is combined with either a MEK inhibitor or an AKT kinase inhibitor, compared with KU-60019 alone. Thus, U87 cells were treated with PD184352, a highly specific and potent MEK1/2 inhibitor (21), with or without KU-60019, and radiosurvival was determined by the trypan blue/FACS assay. Inhibition of MEK/ERK signaling had only a small but significant effect on ionizing radiation-induced killing of these cells (Fig. 6A). Importantly, MEK inhibition did not significantly increase killing by ionizing radiation in combination with KU-60019 compared with KU-60019 alone (Fig. 6A). In fact, when the same response was examined in U1242 cells, treatment with PD184352 alone was slightly radioprotective, and again the combined effect of KU-60019 and PD184352 was not enhanced over KU-60019 alone (Supplementary Fig. S5).

We then examined a possible synergy between ATM and AKT inhibition. Cells were treated with SH-5, a pan-inhibitor

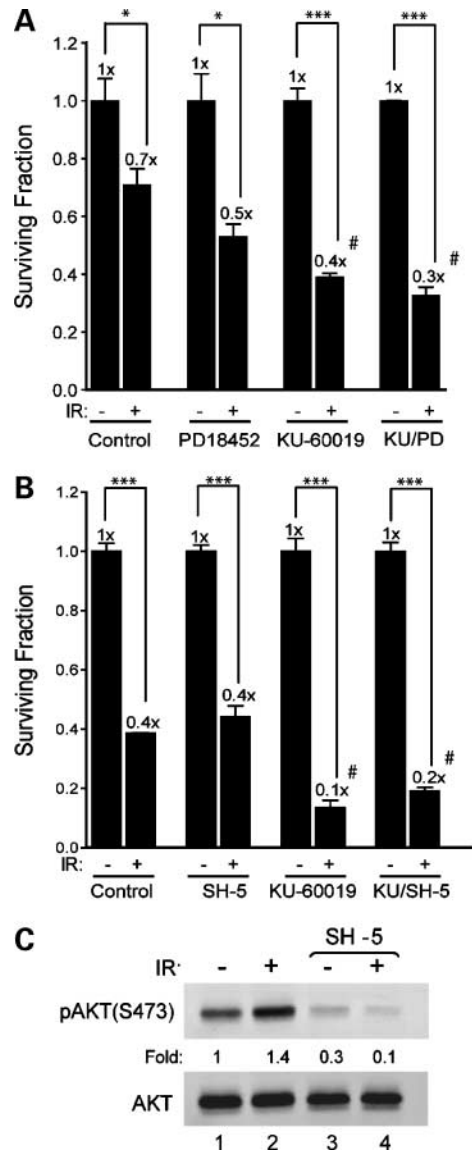


Figure 6. AKT and MEK/ERK signaling are subsets of the ATM signaling network. U87 cells were treated with KU-60019 (1 $\mu\text{mol/L}$), and/or (A) PD184352 (3 $\mu\text{mol/L}$), (B) SH-5 (10 $\mu\text{mol/L}$) for 2 h prior to ionizing radiation (4 Gy). Drugs were removed 16 h post-ionizing radiation. Surviving fractions were determined by trypan blue/FACS assay 7 d post-ionizing radiation. Data points, number of surviving cells plotted as fraction of control (- ionizing radiation); error bars, SE; $n = 3$. Fold (x) depicts relative changes in surviving fractions as compared with drug treated control (- ionizing radiation). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant. #, no significant difference between untreated cells and KU-60019 alone or in combination with PD184352; $P > 0.5$ (A), or SH-5; $P > 0.5$ (B). C, Western blot analysis of extracts from U87 cells treated with SH-5 (10 or 20 $\mu\text{mol/L}$) for 2 h, and then exposed to 5 Gy. Cells were collected for Western blotting 15 min post-ionizing radiation. Fold depicts relative changes in AKT S473 phosphorylation compared with untreated control (-SH-5, - ionizing radiation) after normalizing to total AKT expression levels.

of AKT that binds to AKT and blocks its activation (37). However, when the effects on radiation survival were determined, SH-5 had little effect on ionizing radiation-induced cell killing and was, in fact, radioprotective in both U87 and U1242 cells. When SH-5 was combined with KU-60019 and

ionizing radiation, again no additive effect was observed with KU-60019 in either U87 or U1242 cells (Fig. 6B and Supplementary Fig. S5B). However, we found that SH-5 was able to block AKT phosphorylation by 80% in U87 cells (Fig. 6C). Collectively, these data show that KU-60019 radiosensitization does not increase in combination with inhibitors of either MEK/ERK or AKT signaling that by themselves have little to no effect on glioma cell radiosurvival, suggesting that the effects of KU-60019 on MEK/ERK and AKT signaling on radiosensitization are minor compared with the effects on classical DDR targets and separate from the effects of KU-60019 alone on glioma cell migration, invasion and growth.

Discussion

In this report we have shown that the ATM kinase-specific inhibitor KU-60019 is about 10 times more effective than its predecessor KU-55933 at radiosensitizing human glioma cells. Our results show unambiguously that the phosphorylation of key intracellular targets of the ATM kinase, including p53, H2AX and CHK2, is inhibited or completely abrogated in the presence of low micromolar concentrations of KU-60019. We show that the target for KU-60019 is ATM because normal but not A-T fibroblasts are radiosensitized. Furthermore, 1 $\mu\text{mol/L}$ of KU-60019 almost completely blocked the DDR in cells, a concentration that had no or very little effect on 229 kinases *in vitro*, further supporting the specificity of KU-60019 for the ATM kinase. We also show that KU-60019 reduces phosphorylation of AKT at S473 in glioma and normal fibroblasts but not A-T cells regardless of whether the cells are irradiated, stimulated with insulin, or during normal growth. Our results with KU-60019 agree with those from previous studies showing that A-T cells and normal cells with ATM expression knocked down by small interfering RNA displayed impaired AKT phosphorylation in response to insulin and radiation (13, 14).

Recently, a study by Bozulic et al. reported that DNA-PKcs phosphorylates AKT at S473 in response to radiation, thereby regulating cell survival through p53 and p21 transcriptional control and cell cycle checkpoints that modulate apoptosis (17). In the same study, an experiment addressed whether ATM was also important for this DNA damage response involving DNA-PKcs, but no such involvement was noted (17). However, only ATM $-/-$ mouse embryonic fibroblasts were used and not human cells. We tested normal and ATM $-/-$ mouse embryonic fibroblasts for the ability to modulate S473 AKT phosphorylation in response to radiation, and we also did not find any difference in the AKT response (data not shown). However, our results presented herein using human fibroblasts and glioma cells suggest that ATM is important for controlling AKT phosphorylation. Thus, there could be a difference between how human and mouse cells control DNA damage-induced signaling that influences AKT signaling. There are numerous examples that human and mouse cells process and repair DNA damage differently (38). Importantly, our findings suggest that ATM signaling counteracts AKT phosphorylation by

DNA-PKcs (17) and other kinases acting on AKT in response to insulin.

In human glioma cells, normal fibroblasts, and several other human cancer cell lines (data not shown), KU-60019 reduces S473 AKT phosphorylation regardless of radiation or insulin stimulation. Our findings are in line with the negative regulation of an as of yet unidentified phosphatase acting on p-S473 AKT and p-T308, which is regulated by ATM, i.e., ATM indirectly regulates or needs to directly phosphorylate this phosphatase to keep it inactive. As to the nature of this phosphatase, this is presently unknown. However, there are several candidates able to dephosphorylate AKT, including protein phosphatase 1, protein phosphatase 2A, PTEN, and PHLPP (37, 39–42). Alternatively, ATM could potentially influence DNA-PKcs activity via T2609 phosphorylation, but that is less likely because we still see radiation-induced increases in S473 AKT phosphorylation with KU-60019 present (Fig. 4A). This putative phosphatase is inhibited by low concentrations of okadaic acid suggesting it could be PP1, PP2A, or PP4-6 (32), but more studies are needed to reveal its identity. Although our results are reminiscent of an ATM-regulated phosphatase we cannot at this time rule out alternative mechanisms. Altogether, our results support a role for ATM in regulating AKT phosphorylation, not by increasing phosphorylation but by reducing basal, radiation-, and insulin-induced AKT phosphorylation via a counteracting protein phosphatase.

In an extension of our findings regarding a possible role for ATM in regulating prosurvival AKT and ERK signaling, we also showed that KU-60019 has profound effects on glioma cell migration/motility and invasion *in vitro*. This finding is not surprising because both AKT and ERK have been associated with these processes in a variety of different cell types (for review see ref. 11). The inhibition of basal AKT phosphorylation that we observe with KU-60019 regardless of whether cells are irradiated or not is expected to affect growth, and indeed a slight effect on cell growth was seen after several days in the presence of KU-60019. Both MEK/ERK and AKT have been shown to be upregulated in malignant gliomas and associated with radioresistance and poor prognosis. Inhibitors of both have been explored as potential radiosensitizers with mixed results (33–36). Inhibition of either target is known to reduce tumor growth, but synergy or additive effect with radiation has not been consistent (for review see ref. 10). Our results suggest that in conjunction with KU-60019, neither MEK/ERK nor AKT inhibition is capable of further increasing ionizing radiation-induced cell death over KU-60019 alone. Our findings suggest that that KU-60019 inhibits both AKT and MEK/ERK prosurvival signaling in addition to the phosphorylation of its better-characterized DDR protein targets. The observed additional effects of inhibiting ATM on prosurvival signaling, migration, and invasion, possibly via MEK/ERK and AKT signaling, is an exciting new finding. From a therapeutic standpoint, the inhibition of glioma growth, migration, and invasion *in vitro* by low but yet radiosensitizing concentrations of KU-60019 could perhaps translate into better control of tumor dispersal *in vivo*, a hallmark of malignant glioma (2).

In summary, we have shown herein that KU-60019 is a specific and much improved ATM kinase inhibitor able to radiosensitize human glioma cells in the low micromolar range. Radiosensitization is likely caused by the ability of KU-60019 to inhibit the plethora of ATM phosphorylation targets and upset cell cycle checkpoints, reduce DNA repair, and increase cell death. Furthermore, our results suggest that KU-60019 alone (without radiation) inhibits glioma motility and invasion perhaps acting on the AKT and MEK/ERK pro-survival signaling pathways. Further preclinical testing will address whether some or all of these *in vitro* effects are also seen *in vivo* and whether KU-60019 could be developed into an effective and safe radiosensitizer of malignant glioma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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