The ATM Inhibitor KU-55933 Suppresses Cell Proliferation and Induces Apoptosis by Blocking Akt In Cancer Cells with Overactivated Akt

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Abstract

Aberrant activation of Akt plays a pivotal role in cancer development. ATM, a protein deficient in patients with ataxia-telangiectasia disease, is traditionally considered as a nuclear protein kinase that functions as a signal transducer in response to DNA damage. It has recently been shown that ATM is also a cytoplasmic protein that mediates the full activation of Akt in response to insulin. Our study shows that a specific ATM inhibitor, KU-55933, blocks the phosphorylation of Akt induced by insulin and insulin-like growth factor I in cancer cells that exhibit abnormal Akt activity. Moreover, KU-55933 inhibits cancer cell proliferation by inducing G1 cell cycle arrest. It does so through the downregulation of the synthesis of cyclin D1, a protein known to be elevated in a variety of tumors. In addition, KU-55933 treatment during serum starvation triggers apoptosis in these cancer cells. Our results suggest that KU-55933 may be a novel chemotherapeutic agent targeting cancer resistant to traditional chemotherapy or immunotherapy due to aberrant activation of Akt. Furthermore, KU-55933 completely abrogates rapamycin-induced feedback activation of Akt. Combination of KU-55933 and rapamycin not only induces apoptosis, which is not seen in cancer cells treated only with rapamycin, but also shows better efficacy in inhibiting cancer cell proliferation than each drug alone. Therefore, combining KU-55933 with rapamycin may provide a highly effective approach for improving mammalian target of rapamycin–targeted anticancer therapy that is currently hindered by rapamycin-induced feedback activation of Akt. Mol Cancer Ther; 9(1); 113–25. ©2010 AACR.

Introduction

Akt is a major component of the phosphoinositide 3-kinase (PI3K) signaling pathway that is regulated by insulin and many other growth factors. In normal cells, Akt acts as a signal transducer of PI3K and promotes cell proliferation and cell survival. However, overexpression or overactivation of Akt leads to the development of cancer (1). PI3K consists of an 85-kDa regulatory subunit and a 110-kDa (p110) catalytic subunit. Mutation of PIK3CA, the gene that encodes the p110 subunit of the PI3K, occurs frequently in various types of cancer. Likewise, mutation or loss of PTEN also occurs frequently in cancer. Alteration of both proteins, as well as amplification and mutation of the Akt gene, results in high Akt activity, which leads to the malignant transformation of normal cells (2).

Ataxia-telangiectasia (A-T) is an autosomal recessive inherited disorder characterized by cerebellar ataxia and oculocutaneous telangiectasia (3). Patients with A-T also show symptoms of premature aging, growth retardation, glucose intolerance, and insulin resistance (4). Cell lines derived from A-T patients exhibit hypersensitivity to ionizing radiation (IR), defects in cell cycle control, and increased requirements for serum growth factors (3).

The gene mutated in this disease, ATM (A-T; mutated), encodes a 370-kDa protein kinase that belongs to the PI3K superfamily. The ATM protein kinase phosphorylates p53 and many other cellular targets in response to IR and DNA double-strand breaks (5). Although ATM has previously been reported to be primarily a nuclear protein that functions mainly in controlling cell cycle progression following DNA damage (3), it is now clear that ATM is also present in the cytoplasm and is associated with cytoplasmic proteins and vesicular structures (4, 6).

The cytoplasmic function of ATM has recently started to emerge. Previous studies show that cytoplasmic ATM is an insulin-responsive protein that regulates protein translation through its phosphorylation of a cytoplasmic, translational regulatory protein, 4E-BP1 (4). Following this work, the functional significance of ATM in insulin signaling was further demonstrated by recent findings showing that ATM stimulates Ser473 phosphorylation of Akt and mediates its full activation in response to insulin (7). The involvement of ATM in the insulin/IRS-1/Akt
pathway, as well as its role in the phosphorylation of 4E-BP1, has been further supported by a large-scale proteomic analysis of proteins phosphorylated by ATM (8).

Because Akt is upregulated in many types of cancer cells, it is a valuable target in search for drugs that can be used as chemotherapeutic agents for cancer (9). However, traditional inhibitors of Akt or PI3K are not suitable for treating cancer because of their poor specificity and serious side effects (9). A specific inhibitor of the ATM kinase, known as KU-55933, has been recently discovered (10). KU-55933 has selectivity for ATM that is at least 100-fold greater than that for other related kinases, including the PI3K.

Because ATM mediates the activation of Akt, we tested if KU-55933, by inhibiting ATM, could also prevent the activation of Akt and block the function of its downstream substrates, thereby inhibiting aberrant proliferation of cancer cells. We found that KU-55933 induces cell cycle arrest at the G1 phase by downregulating the synthesis of cyclin D1 in both MDA-MB-453 breast cancer cells and PC-3 prostate cancer cells. In addition, KU-55933 treatment leads to apoptosis under serum starvation conditions. Moreover, addition of KU-55933 to cells treated with rapamycin not only inhibited Akt activation but also caused a significant reduction in cell proliferation rate compared with cancer cells treated with either KU-55933 or rapamycin alone.

### Materials and Methods

#### Chemicals and Antibodies

The insulin and insulin-like growth factor I (IGF-I) were obtained from Sigma. The antibodies against total Akt and cyclin D1 were from Santa Cruz. Antibodies against phospho–glycogen synthase kinase 3β (GSK-3β; Ser9), phospho-Akt (Ser73), phospho-S6 (Ser235/236), and poly ADP-ribose polymerase (PARP) were from Cell Signaling. The anti-β-actin and anti-β-tubulin antibodies were from Sigma. KU-55933 was purchased from Calbiochem. The caspase inhibitor Z-VAD-FMK was from Bachem.

#### Cell Culture and Cell Proliferation Assay by Trypan Blue Staining

MDA-MB-453 and PC-3 cells were purchased from American Type Culture Collection. Sources of A38 and A29 cells were described in ref. (4). These cells were grown in DMEM supplemented with antibiotics and 10% fetal bovine serum. Other cell lines were grown either in DMEM or RPMI according to the supplier’s instructions. For the cell proliferation assay, 6 × 10⁵ cells were seeded into each 25-cm² flask. Every 2 d, cells were trypsinized and stained by trypan blue. Live cells that were not stained with trypan blue were counted.

#### MTT Cell Proliferation Assay

Cells were seeded in a 48-well plate and incubated overnight. Following treatment with KU-55933 and/or rapamycin, the ratio of viable cells in each well was determined using a CellTiter Nonradioactive cell proliferation assay kit (Promega) following the manufacturer’s instructions. Briefly, MTT dye solution in the kit was added to each well and incubated at 37°C for 4 h before the addition of the stop solution. The absorbance at 570 nm was recorded by a microplate reader.

#### SDS-PAGE and Western Blot

Cells were lysed with TGN lysis buffer (11) containing protease inhibitor cocktails (Roche). The protein concentration was measured by the Lowry method. Equal amounts of protein were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane.

#### Flow Cytometry Assay

Following KU-55933 treatment for 3 d, cells were fixed using cold 70% ethanol, treated with RNase, and then stained with propidium iodide. Cells were then subjected to flow cytometry. The cell population at each phase was analyzed by the Modfit 2 software.

#### Reverse Transcription-PCR Analysis

Total cellular RNA was purified from cell lysates by an RNeasy Mini kit (Qiagen) and treated by RNase-free DNase (Qiagen). RNA was then reverse transcribed using the SuperScript first-strand synthesis system (Invitrogen). PCR was done using the Expand High Fidelity PCR system from Roche. The sequence of the forward primer and reverse primer for cyclin D1 is 5′-TGGTTTGCAAGCAGACTTTG-3′ and 5′-ACGTACGCTTCACAAGCTTT-3′, respectively.

#### eIF-4E Pull Down

Cell lysates containing equal amounts of proteins were incubated with M^7-GTP-Sepharose beads overnight at 4°C. The beads were then washed twice with TGN lysis buffer and twice with PBS. The SDS sample loading buffer was added to the beads, and the samples were heated to dissociate the proteins bound to the beads.

#### S35-Labeling Experiments

Cells were serum starved for 24 h. Cells were then incubated with methionine-free medium for 1 h. After the treatment with KU-55933 and insulin, cells were labeled with 90 μCi [35S]-Methionine (PerkinElmer Life Sciences). Cells were then lysed and cyclin D1 was immunoprecipitated using an anti–cyclin D1 antibody. Immunoprecipitated cyclin D1 was then subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and detected by autoradiography.

#### Cell Apoptosis ELISA Assay

The generation of mononucleosomes and oligonucleosomes during apoptosis in cells treated with KU-55933 and/or rapamycin was detected by a Cell Death Detection ELISA kit (Roche) following the manufacturer’s instructions. Briefly, both floating and attached cells were collected, and cell number in each sample was counted.
Cells were lysed gently for 30 min with the buffer provided in the kit. The volume of lysis buffer added to each sample was normalized according to cell number. Cell lysate was then used for the ELISA assay, and the absorbance was detected at 405 nm by a microplate reader.

**Data Analysis**

One-way ANOVA was used to compare means of multiple groups. Comparisons between each two groups were evaluated by a Student’s unpaired t test using Microsoft Office Excel software. The correlation between the phospho-Akt (Ser473) levels and inhibition of cell proliferation by KU-55933 was analyzed using Spearman correlation in the StatMost-32 software. P values of <0.05 were considered statistically significant.

**Results**

**Phosphorylation of Akt at Ser473 in Response to Insulin Fails to Occur in ATM Knockout Murine Embryonic Fibroblast Cells**

The activation of Akt by ATM in response to insulin has been shown in mouse embryonic fibroblast (MEF) cells (7). However, the MEF cell lines used in this study were ATM−/− plus p53−/− (A-T) and p53−/− (control) cells. As a tumor suppressor, p53 can upregulate or downregulate many genes. To confirm that the activation of Akt by ATM is not due to effects of the secondary mutation (p53−/−) in these A-T cells, we compared two isoegenic MEF cell lines derived from normal and ATM knockout mice that do not have the p53 mutation (4).

In normal (A29) MEF cells treated with insulin, Ser473 is readily phosphorylated, whereas Ser473 phosphorylation is almost completely abolished in A-T (A38) MEF cells. In addition, we observed a slight upshift of the Akt protein in normal cells but not in A-T cells (Fig. 1A). Moreover, the phosphorylation of Akt at Ser473 in normal A29 MEF cells is blocked by the specific ATM inhibitor, KU-55933 (Fig. 1B). These results further confirm that ATM mediates Ser473 phosphorylation and the full activation of Akt in response to insulin.

**KU-55933 Inhibits the Phosphorylation of Akt Induced by Growth Factors in Cancer Cells**

MDA-MB-453 is an estrogen receptor–negative/herceptin-resistant breast cancer cell line (12). It has a loss-of-function mutation in the PTEN gene and a gain-of-function mutation in the PIK3CA gene, which lead to overactivation of Akt (2). PC-3 is an androgen-independent PTEN null prostate cancer cell line that shows high levels of basal Akt activity. To test the effect of serum growth factors on Akt phosphorylation in both cell lines, we treated the cells with insulin and IGF-I following serum starvation and observed the increased phosphorylation of Akt at Ser473 (Fig. 1C).

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promotes aberrant cell proliferation in MDA-MB-453 and PC-3 cells. Moreover, KU-55933 markedly inhibited the increase of phospho-Akt at Ser473 (Fig. 1C). Although Akt activation requires phosphorylation at both Ser473 and Thr308 (13, 14), Ser473 phosphorylation was shown to precede the phosphorylation of Thr308 and is in fact a prerequisite for Thr308 phosphorylation (14, 15). Agreeing with
these observations, we also found that KU-55933 inhibited phospho-Akt at Thr308 (Fig. 1C). These results suggest that increased phosphorylation of Akt in response to insulin and IGF-I in MDA-MB-453 and PC-3 cells is also mediated through ATM and that KU-55933 can effectively block the full activation of Akt.

**KU-55933 Suppresses the Proliferation of Cancer Cells**

Akt plays a key role in cell growth and proliferation in response to growth factor stimulation. As shown by MTT assay, KU-55933 treatment inhibited the proliferation of MDA-MB-453 and PC-3 cells in a concentration-dependent manner. The proliferation rate of both cell lines after treatment with 10 μmol/L KU-55933 is ~50% that of control cells (Fig. 2A). Because it was found that at a concentration of 10 μmol/L KU-55933 does not inhibit kinases other than ATM (10), we chose to use 10 μmol/L KU-55933 in our following experiments. Furthermore, long-term (6 or 10 days) inhibition of the proliferation of MDA-MB-453 and PC-3 cells by 10 μmol/L KU-55933 was confirmed by a cell proliferation assay using trypan blue staining (Fig. 2B).

To further determine the relationship between the inhibition of cancer cell proliferation and the inhibition of Akt activity, we examined the correlation between growth inhibition of various cancer cell lines by KU-55933 and their phospho-Akt (Ser473) levels. A panel of cell lines that have varied Akt activities was chosen. These include breast cancer cell lines MDA-MB-453, ZR-75-1, MCF-7, MDA-MB-157, and MDA-MB-231; prostate cancer cell lines PC-3 and LNCaP; osteosarcoma cell line U2OS; and human nontumorigenic mammary epithelial cell line MCF-10A. Phospho-Akt levels of these cell lines were detected by Western blot. The densities of phospho-Akt (Ser473) bands were quantified and normalized to the level of β-actin.

Growth inhibition of these cell lines was measured by MTT assay (Fig. 2C). LNCaP had the highest Akt activity and exhibited strongest inhibition by KU-55933. MDA-MB-453, PC-3, and ZR-75-1 also had high Akt activity, and KU-55933 at 10 μmol/L inhibited ~50% of the cell proliferation in these cells. KU-55933 showed minimal inhibition on MDA-MB-157 and MCF-10A cells, which have weak or no Akt activity (Fig. 2C). The correlation coefficient between levels of phospho-Akt (Ser473) and cell proliferation inhibition by 10 μmol/L KU-55933 was calculated to be 0.8667 (P = 0.0025), which indicates a large positive correlation (Fig. 2D). This result suggests that the inhibition of cell proliferation by KU-55933 is specifically correlated to the inhibition of Akt.

**KU-55933 Treatment Leads to Cell Cycle Arrest at the G1 Phase in Cancer Cells**

One way Akt controls cell proliferation is by stimulating cell cycle progression. Further experiments were done to examine whether KU-55933 can suppress cell proliferation by causing cell cycle arrest. Incubation with KU-55933 resulted in a substantial increase in cell numbers at the G1 phase and a marked decrease at the S phase. As compared with untreated cells, a 2- to 3-fold increase in the G1/S ratio, an indicator of G1 cell cycle arrest (16), was seen in MDA-MB-453 and PC-3 cells treated with KU-55933 (Fig. 3A). This result suggests that KU-55933 inhibits cancer cell proliferation by inducing cell cycle arrest at the G1 phase.

**KU-55933 Treatment Results in the Downregulation of Cyclin D1**

Akt promotes G1 cell cycle progression by upregulating either levels of G1 cyclins or the activity of G1 cyclin-dependent kinases (1). One of these downstream targets is cyclin D1. We thus treated MDA-MB-453 and PC-3 cells with KU-55933 and looked at levels of cyclin D1 protein. As shown in Fig. 3B, cyclin D1 levels decreased dramatically after KU-55933 treatment even in the presence of insulin and IGF-I. Because elevated levels of cyclin D1 are important for the G1 to S transition of the cell cycle, this result suggests that the downregulation of cyclin D1 may account for the G1 cell cycle arrest caused by KU-55933.

In response to serum growth factors, Akt phosphorylates GSK-3β and inactivates its kinase function (1). After treating the cells with insulin and IGF-I, we observed an increase in phosphorylation of GSK-3β at Ser9 in MDA-MB-453 and PC-3 cells. This increased phosphorylation was inhibited after incubating these cancer cells with KU-55933 before insulin and IGF-I treatment (Fig. 3B).

**Downregulation of Cyclin D1 by KU-55933 Does Not Occur through Protein Degradation or Decreased Transcription**

According to a previous report, inhibition of GSK-3β phosphorylation activates GSK, which in turn phosphorylates cyclin D1 and leads to the nuclear export and subsequent degradation of cyclin D1 (17). To test whether this is the case in our study, we treated MDA-MB-453 and PC-3 cells with ALLN, a proteasome inhibitor, to block protein degradation. ALLN treatment effectively blocked the degradation of cyclin D1 in both cell lines. To our surprise, however, in cells treated with KU-55933, levels of cyclin D1 still decreased in the presence of ALLN, indicating that the downregulation of cyclin D1 by KU-55933 did not occur posttranslationally through protein degradation (Fig. 3C).

In addition, previous studies have shown a positive correlation between overexpression of β-catenin and enhanced transcription of cyclin D1 mRNA in certain types of cancer cells (1). In these cells, the dephosphorylation and activation of GSK-3β lead to the increased degradation and cytoplasm sequestration of β-catenin. Sequestered β-catenin is unable to enter the nucleus to activate cyclin D1 gene transcription (18, 19). To determine whether KU-55933 causes decreased cyclin D1 transcription, total cellular RNA was isolated from MDA-MB-453 and PC-3 cells treated with KU-55933. Reverse transcription-PCR results indicate that cyclin D1 mRNA levels did
not change after KU-55933 treatment, suggesting that cyclin D1 is not downregulated by KU-55933 at the level of transcription (Fig. 3D).

**KU-55933 Suppresses Cyclin D1 Translation Initiation by Inhibiting Phosphorylation of 4E-BP1**

It is known that cyclin D1 expression is also regulated by protein translation. Specifically, cyclin D1 has been considered a potential downstream target of 4E-BP1 (20). As a suppressor of protein translation, hypophosphorylated 4E-BP1 inhibits cap-dependent translation by binding to eIF-4E, whereas phosphorylation of 4E-BP1 in response to growth factors releases eIF-4E from 4E-BP1 to facilitate cap-dependent translation. Phosphorylation of 4E-BP1 involves multiple phosphorylation sites, and both mammalian target of rapamycin (mTOR; a downstream target of Akt) and ATM phosphorylate 4E-BP1 in response to insulin (4, 21).

To test whether KU-55933 inhibits growth factor–mediated 4E-BP1 phosphorylation, we detected total 4E-BP1 in MDA-MB-453 and PC-3 cells treated with KU-55933 (10 μmol/L) for 3 d. After treatment, cells were collected and subjected to flow cytometry analysis after they were fixed with 70% ethanol and stained with propidium iodide. The results were analyzed by the Modfit 2 software.

**Figure 3.** A, KU-55933 arrests the cell cycle at the G1 phase. MDA-MB-453 and PC-3 cells were treated with KU-55933 (10 μmol/L) for 3 d. After treatment, cells were collected and subjected to flow cytometry analysis after they were fixed with 70% ethanol and stained with propidium iodide. The results were analyzed by the Modfit 2 software.
Next, we isolated the eIF-4E/4E-BP1 complex from MDA-MB-453 and PC-3 cells that were treated with KU-55933 and further examined the interaction between 4E-BP1 and eIF-4E. We observed increased amounts of 4E-BP1 bound to eIF-4E in cells treated with KU-55933 compared with untreated control cells (Fig. 4B). These results clearly show that KU-55933, by inhibiting the phosphorylation of 4E-BP1, may suppress growth factor–mediated protein translation initiation.

To test whether cyclin D1 is downregulated by the decreased protein translation caused by KU-55933, newly synthesized cyclin D1 was measured by $^{38}$S labeling of MDA-MB-453 and PC-3 cells. Compared with control cells, increased de novo translation of cyclin D1 was seen following insulin treatment. However, pretreatment of cells with KU-55933 resulted in a marked decrease of newly translated cyclin D1 protein compared with cells treated with insulin alone (Fig. 4C). These results suggest that inhibition of cyclin D1 translation is the underlying mechanism for the downregulation of cyclin D1 by KU-55933.

**Treatment with KU-55933 During Serum Starvation Leads to Increased Apoptosis**

In addition to stimulating cell proliferation, Akt is also well documented for promoting cell survival in the presence of growth factors. It is conceivable that inhibition of Akt during serum deprivation may lead to apoptosis. We thus further investigated whether treatment with KU-55933 causes cell apoptosis when serum growth factors in the medium are depleted. MDA-MB-453 and PC-3 cells were incubated with KU-55933 for 2 h. Total cellular RNA was purified and reverse transcription–PCR was done as described in Materials and Methods. The midpoint for linearity of the exponential phase of amplification of the cyclin D1 mRNA was determined to be 25 cycles. The PCR products were resolved in a 1.0% agarose gel stained with ethidium bromide. The results in A to D are representative of three individual experiments.
A dramatic increase in cleaved PARP levels (Fig. 5A). These results suggest that treatment with KU-55933 during serum starvation leads to enhanced apoptosis in MDA-MB-453 and PC-3 cells.

Cell apoptosis caused by KU-55933 treatment was also confirmed by the observation that KU-55933-induced cleavage of PARP was completely abrogated through the addition of the caspase inhibitor Z-VAD-FMK (Fig. 5B). The apoptosis induced by KU-55933 was further detected by a cell death ELISA assay. Neither MDA-MB-453 nor PC-3 cells exhibited increased apoptosis following a 2-day serum starvation compared with nonstarved cells (Fig. 5C). However, after treatment with KU-55933 for 2 days in a serum-free medium, apoptotic DNA fragmentation increased 2-fold in MDA-MB-453 cells and over 3-fold in PC-3 cells compared with untreated serum-starved (control) cells (Fig. 5C).

Combination of KU-55933 and Rapamycin Induces Apoptosis That Is Not Seen in Cancer Cells Treated with Rapamycin Alone

mTOR is a downstream target of Akt that phosphorylates multiple protein translation factors, including 4E-BP1 and the p70S6 kinase. Suppression of cancer cell growth has been achieved by inhibiting mTOR with the mTOR inhibitor rapamycin (22, 23). However, the inhibition of mTOR causes feedback activation of Akt (24, 25), which hinders treatment outcome in clinical trials that have used derivatives of rapamycin as chemotherapeutic agents (22, 23).

Because KU-55933 inhibits Ser473 phosphorylation of Akt, we tested the effect of KU-55933 on the Akt activation caused by rapamycin. Results in Fig. 6A indicate that feedback activation of Akt caused by rapamycin in MDA-MB-453 cells was completely abolished by KU-55933. Furthermore, although rapamycin alone failed to induce apoptosis of these cancer cells, treatment of cells...
with KU-55933 plus rapamycin led to cell death by triggering the cleavage of PARP (Fig. 6A). These results were also confirmed by a cell death ELISA assay, which indicate a 3-fold increase in apoptotic DNA fragmentation in MDA-MB-453 cells treated with both KU-55933 and rapamycin compared with cells treated with only rapamycin (Fig. 6B). Treatment of PC-3 cells with rapamycin did not cause feedback activation of Akt. However, rapamycin alone still failed to induce cell apoptosis compared with control cells (Fig. 6A and B). Cell apoptosis, as indicated by cleaved PARP and increased DNA fragmentation, was only observed after addition of KU-55933,

Figure 5. KU-55933 (KU) treatment in a serum-free medium induces apoptosis in MDA-MB-453 and PC-3 cells. A, subconfluent cells were treated with KU-55933 (10 μmol/L) for 48 h in a serum-free medium. Both floating and attached cells were collected after treatment. Cells were lysed by TGN buffer, and cell lysates were subjected to SDS-PAGE and immunoblotting. PARP, cleaved-PARP, phospho-Akt at Ser473 and Thr308, and β-actin were detected. B, cells were pretreated with a general caspase inhibitor Z-VAD-FMK (VAD; 50 μmol/L) for 1 h in a serum-free medium and then treated with KU-55933 (10 μmol/L) for 48 h. Cells were harvested and lysed as described above. PARP, cleaved-PARP, and β-actin were detected. The results in A and B are representative of three individual experiments. C, cells were treated with KU-55933 (10 μmol/L) for 48 h in a serum-free medium. Both floating and attached cells were collected after treatment. Cell death ELISA assays were then performed as described in Materials and Methods. Columns, mean of three replicates (*, P < 0.05); bars, SEM.
Figure 6. The combination of KU-55933 (KU) with rapamycin leads to apoptosis that is not seen in cells treated with rapamycin alone. A, MDA-MB-453 and PC-3 cells were exposed to rapamycin (Rap; 20 nmol/L) or the combination of KU-55933 (10 μmol/L) and rapamycin (Rap+KU; 20 nmol/L) for 48 h in a serum-free medium. Both floating and attached cells were collected after treatment. Cells were lysed by TGN buffer, and cell lysates were subjected to SDS-PAGE and immunoblotting. Phospho-Akt at Ser473 and Thr308, phospho-S6 at Ser235/236, PARP, cleaved-PARP, and β-tubulin were detected. B, MDA-MB-453 and PC-3 cells were exposed to KU-55933 (10 μmol/L), rapamycin (20 nmol/L), or the combination of KU-55933 (10 μmol/L) and rapamycin (20 nmol/L) for 48 h in a serum-free medium. Both floating and attached cells were collected for detection of apoptosis by the cell death ELISA kit as described in Materials and Methods. Columns, mean of three replicates (*, P < 0.05; **, P < 0.01); bars, SEM. C, the combination of rapamycin with KU-55933 is more effective in inhibiting cell proliferation than KU-55933 or rapamycin alone. Cells were seeded in a 48-well plate and were treated with KU-55933 (10 μmol/L), rapamycin (20 nmol/L), or rapamycin (20 nmol/L) plus KU-55933 (10 μmol/L) for 2 d in a serum-free medium. Cell proliferation rate was then detected by MTT assay. Columns, mean of absorbance from three separate samples (*, P < 0.05; **, P < 0.01); bars, SEM.
Akt Inhibition by ATM Inhibitor KU-55933

which completely inhibited the residual Akt activity (Fig. 6A and B).

Rapamycin and its analogues exhibit strong cytostatic effects by inhibiting G1 cell cycle progression in cancer cells (26, 27). KU-55933, in addition to its cytostatic activity, is able to induce apoptosis by inhibiting Akt phosphorylation. We therefore further tested the overall effect of the combination of KU-55933 and rapamycin on cancer cell proliferation by MTT assay, a measurement that reflects both cell cycle arrest and the induction of apoptosis caused by cancer chemotherapeutic agents (28). As seen in Fig. 6C, although rapamycin or KU-55933 alone has limited antiproliferative effect on MDA-MB-453 cells and PC-3 cells, combined treatment of these cancer cells with KU-55933 and rapamycin led to a significant reduction in cell proliferation rate, suggesting a cooperative inhibitory effect of KU-55933 and rapamycin on cancer cell proliferation.

Discussion

The majority of studies on ATM focus on its nuclear function, which is to trigger the DNA damage response by phosphorylating its downstream substrates and causing cell cycle arrest. Therefore, KU-55933 has been used either as a tool for studies of the function of ATM in cellular response to DNA damage or as a radiosensitizer in cancer radiotherapy (10). In contrast, our study using KU-55933 targets the cytoplasmic function of ATM in mediating Akt activation in response to insulin and IGF-I. Our results provide the first evidence that KU-55933, through inhibition of ATM and its downstream target Akt, inhibits cell proliferation by inducing cell cycle arrest and apoptosis in cancer cells that exhibit abnormal Akt activation.

Our results from ATM knockout MEF cells have confirmed a previous report indicating that ATM mediates the full activation of Akt through upregulation of phosphorylation of Akt at Ser473 (7). We further show that ATM activates Akt in response to insulin and IGF-I by observing that KU-55933 inhibits insulin and IGF-I–stimulated Akt phosphorylation at both Ser473 and Thr308. It should be noted that higher doses of KU-55933 are required to inhibit phospho-Akt at Thr308 than phospho-Akt at Ser473 (Supplementary Fig. S1). Compared with a previous finding showing that a PI3K inhibitor has similar inhibitory effect on both phosphorylation sites of Akt (29), this result suggests that KU-55933 may specifically inhibit PDK2, the proposed upstream kinase of Ser473, rather than PDK1, the direct upstream kinase of Thr308 of Akt.

The activation of Akt by ATM occurs in the cytoplasm through a growth factor–mediated signaling pathway. This finding agrees with many of the recently discovered cytoplasmic functions of ATM, including its phosphorylation of 4E-BP1 (4). In addition, ATM has been shown to promote neuronal survival in an insulin-dependent manner in differentiated neuron-like SH-SY5Y cells (11). More recently, ATM was found to participate in insulin-regulated glucose uptake in muscle cells (30). However, ATM cannot phosphorylate Akt in vitro (7). It is therefore possible that ATM may act as an upstream kinase of one of the several candidate kinases for PDK2 that can directly phosphorylate Akt at Ser473 (14, 31, 32).

Several members of the PI3K family are known to regulate Akt phosphorylation at Ser473. A recently discovered mTOR-rictor complex (14) or mTORC2 is a candidate as a potential upstream kinase of Akt at Ser473. Different from the typical mTOR (also called mTORC1) that binds to a protein partner raptor and is sensitive to rapamycin, the rictor-containing complex (also called mTORC2) does not seem to be rapamycin sensitive, and its cellular function is just beginning to be understood (14, 33). It was shown that mTORC2 can phosphorylate Ser473 of Akt in response to insulin in cultured cells (14). It will be interesting to determine whether ATM is the upstream kinase of mTORC2.

DNA-PK, another member of the PI3K superfamily, was implicated in the activation of Akt following IR (32). Interestingly, DNA-PK was also shown to phosphorylate Akt Ser473 in vitro (34). Furthermore, DNA-PK–deficient cells fail to elevate phospho-Akt (Ser473) levels in response to insulin. In DNA-PK–proficient cells, insulin-stimulated Akt phosphorylation can be abolished by DNA-PK knockdown (34). A recent finding indicating that DNA-PK promotes fatty acid synthesis in response to insulin also highlights the involvement of DNA-PK in an insulin signaling pathway (35). We detected the phosphorylation of Akt at Ser473 in response to insulin and IGF-I after the treatment of MDA-MB-453 and PC-3 cancer cells with DNA-PK inhibitor NU7026 and found that NU7026 slightly inhibited the phosphorylation of Akt in these cells (Supplementary Fig. S2). Because ATM was previously shown to be an upstream kinase of DNA-PK in response to IR (36), it is possible that ATM may mediate insulin-stimulated Akt phosphorylation at least partially through activation of DNA-PK.

The activation of the PI3K/Akt pathway is an early event in carcinogenesis and promotes cancer progression to an aggressive stage by stimulating cell growth, cell proliferation, and cell survival (1). In addition, the overactivation of Akt accounts for the resistance of cancer cells to chemotherapy and immunotherapy, and the activation of this pathway is a poor prognostic factor in cancer (22). However, several Akt inhibitors tested in multiple clinical trials, such as perifosine and triciribine, have serious side effects and limited efficacy against cancer with aberrant Akt activity (37–39). There is therefore an urgent need to find new chemotherapeutic drugs that can effectively target overactivated Akt in these cancers.

KU-55933 is a very specific inhibitor of ATM. Therefore, in contrast to traditional inhibitors of Akt, KU-55933 may provide more efficient inhibition of Akt activity with fewer side effects. By comparing the antiproliferation effect of KU-55933 in a panel of cell lines with different levels of Akt activity, we found that treatment of KU-55933 resulted in a higher degree of inhibition of cell proliferation in cell lines with higher Akt activity. A strong positive...
correlation exists between Akt activity and the antiproliferation effect of KU-55933. These results suggest that KU-55933 specifically targets cancer cells with a high basal level of Akt activity and inhibits cancer cell proliferation mainly through the inhibition of Akt.

Cyclin D1 is frequently overexpressed in a variety of tumors (40). Our observation that KU-55933 arrests the cell cycle at G1 is supported by the finding that KU-55933 downregulates cyclin D1. We have shown that KU-55933 does not facilitate degradation or decrease the transcription of cyclin D1. Our findings, however, are the first to show that the inhibition of ATM is linked to the downregulation of cyclin D1 translation. Our results show that KU-55933 inhibits the phosphorylation of 4E-BP1 and facilitates binding between 4E-BP1 and eIF-4E, suggesting that the decreased cyclin D1 translation is mediated by dephosphorylation of 4E-BP1.

mTOR phosphorylates 4E-BP1 at Thr36 and Thr45 in vivo (21) and is also known to be downstream of Akt (41). Therefore, KU-55933 may inhibit 4E-BP1 phosphorylation at these two sites. In addition, ATM itself can also phosphorylate 4E-BP1 in vivo at Ser111 in response to insulin, which may cooperate with mTOR-mediated 4E-BP1 phosphorylation and also lead to enhanced protein translation efficiency (4). It is likely that KU-55933 inhibits cyclin D1 translation through the inhibition of both Akt/mTOR and ATM-mediated 4E-BP1 phosphorylation.

Growth factor depletion can switch cell growth arrest to cell death (42). Consistent with the fact that Akt promotes cell survival in the presence of growth factors, KU-55933 treatment was found to induce apoptosis during serum starvation. This is important for cancer therapy, as malignant tumor cells can grow even under serum-starved conditions. In fact, many cancer cells have lost the ability to undergo apoptosis in response to growth factor deprivation. It however remains unclear which pathway downstream of Akt is involved in apoptosis induction. Activity of FOXO1, a member of the Forkhead proapoptotic transcription factors and a direct target of Akt, is inhibited by ATM (7). It will be important to determine whether KU-55933 induces apoptosis through inhibition of the Akt/FOXO1 axis or other Akt-mediated cell survival pathways.

Combination therapy with new and existing drugs has become a promising approach to treat cancer and diseases with drug resistance (43). Rapamycin is a classic drug that has been shown to suppress the growth of cancer cells through inhibition of mTOR. However, the feedback activation of Akt caused by rapamycin limits the clinical application of rapamycin and its analogues, most likely due to enhanced survivability of the cell caused by rapamycin-mediated Akt activation (22). In contrast, KU-55933 not only has a cytostatic effect (by inhibiting protein translation) on cancer cell proliferation, but also exhibits a strong cytotoxic effect that was not seen in cancer cells treated with rapamycin.

Because rapamycin and its analogues have limited efficacy in a variety of tumor types due to their feedback activation of Akt, it was proposed that combinational therapy of rapamycin with PI3K/Akt inhibitors may be a more effective option for cancer treatment (22). Our work shows that the feedback activation of Akt caused by rapamycin was completely abolished by KU-55933 in MDA-MB-453 cells. As compared with rapamycin treatment alone, this combinational treatment also triggered apoptosis in MDA-MB-453 cells. Even in PC-3 cells where no feedback activation of Akt was observed, rapamycin treatment still could not induce apoptosis, possibly due to the existing Akt activity in the cancer cells. The presence of KU-55933 is essential to further inhibit the Akt activity, thereby inducing cell death. Moreover, combination of KU-55933 with rapamycin not only inhibited Akt activation but also caused a further reduction in cell proliferation rate compared with cancer cells treated with either KU-55933 or rapamycin alone. These results suggest that KU-55933 or its analogues may be combined with rapamycin to significantly improve mTOR-targeted anticancer therapy.

Mutations in the PI3K pathway occur frequently in human cancers, second only to the mutation rate of the p53 tumor suppressor (2). Because of the strong cytostatic and cytotoxic effects of KU-55933 against cancer cell proliferation and its high specificity toward ATM and Akt, KU-55933 or its analogues, either used alone or in combination with other chemotherapeutic agents such as rapamycin, may become a novel and highly effective chemotherapeutic agent for treating many types of aggressive cancer against that current chemotherapy and immunotherapy have limited efficacy.

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