Chemosensitization of Cancer Cells by KU-0060648, a Dual Inhibitor of DNA-PK and PI-3K

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Abstract

DNA double-strand breaks (DSB) are the most cytotoxic lesions induced by topoisomerase II poisons. Nonhomologous end joining (NHEJ) is a major pathway for DSB repair and requires DNA-dependent protein kinase (DNA-PK) activity. DNA-PK catalytic subunit (DNA-PKcs) is structurally similar to PI-3K, which promotes cell survival and proliferation and is upregulated in many cancers. KU-0060648 is a dual inhibitor of DNA-PK and PI-3K in vitro. KU-0060648 was investigated in a panel of human breast and colon cancer cells. The compound inhibited cellular DNA-PK autophosphorylation with IC50 values of 0.019 μmol/L (MCF7 cells) and 0.17 μmol/L (SW620 cells), and PI-3K–mediated AKT phosphorylation with IC50 values of 0.039 μmol/L (MCF7 cells) and more than 10 μmol/L (SW620 cells). Five-day exposure to 1 μmol/L KU-0060648 inhibited cell proliferation by more than 95% in MCF7 cells but only by 55% in SW620 cells. In clonogenic survival assays, KU-0060648 increased the cytotoxicity of etoposide and doxorubicin across the panel of DNA-PKcs–proficient cells, but not in DNA-PKcs–deficient cells, thus confirming that enhanced cytotoxicity was due to DNA-PK inhibition. In mice bearing SW620 and MCF7 xenografts, concentrations of KU-0060648 that were sufficient for in vitro growth inhibition and chemosensitization were maintained within the tumor for at least 4 hours at nontoxic doses. KU-0060648 alone delayed the growth of MCF7 xenografts and increased etoposide-induced tumor growth delay in both SW620 and MCF7 xenografts by up to 4.5-fold, without exacerbating etoposide toxicity to unacceptable levels. The proof-of-principle in vitro and in vivo chemosensitization with KU-0060648 justifies further evaluation of dual DNA-PK and PI-3K inhibitors. Mol Cancer Ther; 11(8); 1789–98. ©2012 AACR.

Introduction

DNA double-strand breaks (DSB) are the principal cytotoxic lesions induced by topoisomerase II poisons, such as etoposide and doxorubicin (1), which are widely used in the treatment of a variety of human solid malignancies and leukemia (2). Unrepaired DSBs trigger cell-cycle arrest and/or cell death, and it is thought that even a single DSB may be enough to induce cell death (1, 3).

As DSBs may also be generated endogenously or by environmental toxins, cells have developed complex mechanisms to repair DSBs for survival. In mammalian cells, homologous recombination (HR) and nonhomologous end-joining (NHEJ) are the major DSB repair pathways (4, 5). The principal factor determining the choice of mechanism is in which cell-cycle phase the DSBs occur, or are recognized (6, 7). HR is dependent on the proximity of the sister chromatid and can therefore only take place in late S- and G2 phase. In contrast, NHEJ rejoins broken DNA ends without reference to a second template and is predominant during G1 or G0 phase, but also operational during other cell-cycle phases (8).

DNA DSB repair, while essential for survival, may constitute a mechanism of therapeutic resistance to certain DNA-damaging agents. Consequently, cells defective in DSB repair by virtue of the inactivation of a component of the NHEJ pathway are highly sensitive to ionizing radiation (IR) and topoisomerase II poisons (9–16). Conversely, overexpression of DNA-PKcs, a key component of the NHEJ pathway, can accelerate the repair of IR-, etoposide-, and doxorubicin-induced DSBs, thus conferring resistance to these agents (17, 18). In the clinical setting, DNA-PK protein expression correlates with resistance to etoposide in human chronic lymphocytic leukemia samples (19). Therefore, the inhibition of DNA-PK is an attractive approach for modulating resistance to therapeutically induced DNA DSBs.

DNA-PK is a member of the phosphoinositide 3-kinase (PI-3K)-related protein kinase family of enzymes. PI-3K regulates a wide range of cellular processes, including...
those central to cell growth and survival. Aberrant activation of the PI-3K signalling pathway is common in many human cancers (20–22). Therefore, the inhibition of PI-3K is also an attractive target for the development of cancer therapies.

Consistent with the structural similarities between PI-3K and DNA-PKcs, the PI-3K inhibitor LY294002 was identified as a competitive inhibitor of DNA-PK (23). LY294002 has been reported to retard DSB repair and enhance the cytotoxicity of IR, which despite the lack of specificity, has largely been attributed to the inhibition of DNA-PKcs (24). Ongoing efforts within our research group using LY294002 as a starting pharmacophore for chemistry gave rise to the discovery of more potent and specific inhibitors of DNA-PKcs (15, 25). Our group identified NU7441 as a potent inhibitor of DNA-PK (26–28), and continuing compound development has identified NU7441 as a potent inhibitor of DNA-PK and specific inhibitors of DNA-PKcs (15, 25). Our group of DNA-PKcs (24). Ongoing efforts within our research group using LY294002 as a starting pharmacophore for chemistry gave rise to the discovery of more potent and specific inhibitors of DNA-PKcs (15, 25). Our group identified NU7441 as a potent inhibitor of DNA-PK (26–28), and continuing compound development has identified NU7441 as a potent inhibitor of DNA-PK and specific inhibitors of DNA-PKcs (15, 25). Our group of DNA-PKcs (24). Ongoing efforts within our research group using LY294002 as a starting pharmacophore for chemistry gave rise to the discovery of more potent and specific inhibitors of DNA-PKcs (15, 25). Our group identified NU7441 as a potent inhibitor of DNA-PK (26–28), and continuing compound development has identified NU7441 as a potent inhibitor of DNA-PK (23).

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Determination of cellular activity of KU-0060648 against DNA-PK and PI-3K

DNA-PK autophosphorylation was determined in cells exposed to a range of concentrations of KU-0060648 for 1 hour before X-irradiation (10 Gy). Cell lysates were prepared 30 minutes later using Phosphosafe Extraction Reagent (Merck) according to the manufacturer’s instructions. Levels of DNA-PKcs autophosphorylation at Ser2056 (31), relative to unphosphorylated DNA-PKcs, were determined by Western blotting. To determine PI-3K activity, cells were exposed to a range of concentrations of KU-0060648 for 1 hour before a 30-minute treatment with 50 ng/mL insulin-like growth factor-I (IGF-I; Calbiochem, Merck Biosciences). The levels of PI-3K–dependent AKT phosphorylation (Ser473) (ref. 32) relative to unphosphorylated AKT were determined by Western blotting.

Gel electrophoresis was conducted with Tris-acetate 3% to 8% (v/v) polyacrylamide gradient gels (Bio-Rad). Samples transferred onto Amersham Hybond C Nitrocellulose Membrane (GE Healthcare Life Sciences) were probed with primary antibodies against DNA-PKcs (H-163; 1:1,000; Santa Cruz Biotechnology), phosphorylated Ser2056 DNA-PKcs (ab20407; 1:1,000; Abcam), AKT (C67E7; 1:500; New England Biolabs), phosphorylated Ser473 AKT (9252; 1:1,000; New England Biolabs), and actin (Ab-1; 1:10,000; Calbiochem, Merck Biosciences). Anti-rabbit or anti-mouse (actin) horseradish peroxidase–linked secondary antibodies (1:1,000; Dako) and enhanced chemiluminescence reagent (GE Healthcare Life Sciences) were used for detection, followed by image acquisition and densitometry with a Fuji LAS-3000 Luminescent Image Analyzer (Raytek).

The increase in phosphorylated protein above the baseline levels of unstimulated cells was measured, and the...
level detected in extracts from KU-0060648–treated cells expressed as a percentage of the increase in control cells. The mean values of 3 independent experiments were plotted as a sigmoidal dose-response curve and the IC_{50} values calculated using GraphPad Prism software (GraphPad Software, Inc.).

**Cytotoxicity and growth inhibition studies**

Cytotoxicity was measured by clonogenic assays. Cells grown in 6-well plates were exposed to etoposide or doxorubicin, with or without KU-0060648 (1 μmol/L) for 16 hours, before harvesting and seeding into 10-cm diameter Petri dishes, in drug-free medium. Colonies were stained with crystal violet after 10 to 14 days and counted with an automated colony counter (ColCount, Oxford Optronics Ltd.). Cell growth inhibition, following 5-day continuous exposure to KU-0060648, was determined by the sulforhodamine B (SRB) assay, as described previously (33). The GI_{50} is the concentration causing 50% cell growth inhibition.

**KU-0060648 plasma pharmacokinetics following different routes of administration**

All in vivo experiments were reviewed and approved by the relevant Institutional Animal Welfare Committees and carried out according to the National Law. We determined the plasma pharmacokinetics of KU-0060648 following administration i.v., i.p., or orally at 10 mg/kg to female Balb/C mice (Charles River). KU-0060648 was formulated in a vehicle of equimolar phosphoric acid, made up to volume with sterile saline and at final pH 5. Mice were killed at intervals up to 360 minutes after KU-0060648 administration, and plasma concentrations of KU-0060648 were determined by liquid chromatography/tandem mass spectrometry (LC/MS-MS) analysis, as previously described (27).

**KU-0060648 distribution to tumor xenografts**

Female athymic mice (CD1-nu/nu; Charles River) were maintained and handled in isolators under specific pathogen-free conditions for tissue distribution and efficacy studies. KU-0060648 (12.5 mg/kg i.v.) was administered to MCF7 or SW620 tumor–bearing mice (650 mm^3), which were killed 60 or 240 minutes later. Tumors were excised and homogenized in PBS (1:3 w/v), with a stirrer macsercarator homogenizer (IKG Werke GmbH & Co.), in 10-second bursts, on ice. Plasma and tumor KU-0060648 concentrations were determined by LC/MS-MS analysis, as previously described (27).

**DNA-PK ex vivo pharmacodynamic assay**

KU-0060648 at 2.5 or 25 mg/kg or vehicle alone was administered to SW620 tumor–bearing mice i.v.. After 1 or 4 hours, animals were killed, and tumors were excised and homogenized. DNA-PK activity within tumor homogenates was determined by measuring the DNA-PK-dependent phosphorylation of a p53 peptide substrate (Ser^39), using ELISA assay, as described previously (34).

**Antitumor efficacy study**

Mice bearing SW620 or MCF7 xenografts subcutaneously (n = 5/group) were treated when tumors were palpable (~ 5 x 5 mm^2, 8–10 days after implantation). Animals received normal saline i.p. once daily (control), single-agent KU-0060648 10 mg/kg i.p. twice daily for either 5 days in SW620 tumor–bearing, or 14 days in MCF7 tumor–bearing mice, with doses on each day 8 hours apart, or etoposide phosphate once daily i.p. (11.35 mg/kg in saline, equivalent to 10 mg/kg free etoposide, i.p for five days). For combinations, KU-0060648 was administered i.p. once or twice daily for 5 days (SW620) or once daily for 14 days (MCF7), with the first dose immediately before etoposide phosphate.

Tumor volume was calculated from a 2-dimensional electronic caliper (Mitutoyo) measurements using the equation \( a^2 \times b/2 \), where \( a \) is the smallest measurement and \( b \) the largest. Data are presented as median relative tumor volume (RTV), where the tumor volume on the initial day of treatment (day 0) is assigned an RTV value of 1.

**Results**

**Cellular activity of KU-0060648 against DNA-PK and PI-3K**

We used a cell-based assay to determine the inhibitory activity of KU-0060648 against DNA-PK and PI-3K in SW620 and MCF7 cells. IR treatment induced an approximately 20-fold increase in DNA-PK autophosphorylation levels, which was inhibited by KU-0060648 in a concentration-dependent manner, with an IC_{50} value of 0.019 μmol/L in MCF7 cells and 0.17 μmol/L in SW620 cells (Supplementary Fig. S2). IGF-I treatment caused an approximately 8-fold increase in AKT phosphorylation, in which MCF7 cells were inhibited in a KU-0060648 concentration-dependent manner, with an IC_{50} value of 0.039 μmol/L. In contrast, the previous lead inhibitor, NU7441, was approximately 20 times more potent against DNA-PK (IC_{50} = 0.2 ± 0.03 μmol/L) than PI-3K (IC_{50} = 3.9 ± 3.5 μmol/L) in MCF7 cells. KU-0060648 was virtually inactive against PI-3K in SW620 cells in which the IC_{50} for the inhibition of AKT phosphorylation was more than 10 μmol/L (with only 42% inhibition at this concentration; Supplementary Fig. S2), suggesting that the inhibition of PI-3K by KU-0060648 may be cell line–dependent. In a control experiment, the PI-3K inhibitor ZSTK474 [2-(2-difluoromethylbenzimidazol-1-yl)-4,6-dimorpholino-1,3,5-triazine; ref. 35] was used to show that it is possible to inhibit AKT Ser^{39} phosphorylation using a PI-3K inhibitor in SW620 cells. Unlike KU-0060648, the compound did not cause such pronounced cell-specific differences in inhibition of AKT phosphorylation, with IC_{50} values of 0.2 μmol/L in SW620 cells and 0.02 μmol/L in MCF7 cells (Supplementary Fig. S3).

**Chemopotentiation by KU-0060648 in vitro**

We investigated the DNA-PK dependence of chemosensitization by KU-0060648 in mammalian and human...
cells with and without DNA-PK function. The DNA-PKcs–deficient V3 cells were approximately 3-fold more sensitive to etoposide than the DNA-PKcs–proficient V3-YAC cells (Fig. 1A and B). Similarly, the DNA-PKcs–deficient M059J cells were approximately 2.5-fold more sensitive to etoposide than the DNA-PKcs–proficient M059-Fus-1 cells (Fig. 1C and D). KU-0060648 alone caused less than 15% reduction in cell viability in V3 and V3-YAC cells and less than 5% reduction in cell viability in M059J and M059-Fus-1 cells, but enhanced the cytotoxicity of etoposide by more than 13-fold in V3-YAC cells and 4-fold in M059-Fus-1 cells, compared with only 2.5-fold and 1.1- to 1.5-fold in V3 and M059J cells, respectively (Fig. 1A-D). There was an even more profound difference in the sensitivities of M059J and M059-Fus-1 cells to doxorubicin. M059J cells were approximately 23-fold more sensitive to doxorubicin than M059-Fus-1 cells. KU-0060648 enhanced the cytotoxicity of doxorubicin by up to 32-fold in M059-Fus-1 cells (depending on the doxorubicin concentration), but only 1.4-fold in M059J cells (Fig. 1E and F). We confirmed that M059J and M059-Fus-1 cells have comparable PI-3K activity levels and that AKT phosphorylation is inhibited to the same extent by KU-0060648 and ZSTK474 in each of the cell lines (Fig. 1G). Therefore chemosensitization of topoisomerase II poisons by KU-0060648 is largely due to DNA-PK inhibition.

We investigated growth inhibition by KU-0060648 in a panel of human breast (MCF7, T47D, and MDA-MB-231) and colon (LoVo and SW620) cancer cells. DNA-PK expression was confirmed in each of the cell lines, and inhibition of DNA DSB repair by KU-0060648 was shown in SW620 cells (Supplementary Fig. S4). Exposure to 1 µmol/L KU-0060648 for 5 days resulted in more than 50% inhibition of cell growth in all cell lines (Fig. 2A). The greatest effect on growth inhibition was observed in LoVo and MCF7 cells, in which total cell growth over 5 days was only 10% and 4% of that of DMSO-treated controls, respectively. KU-0060648 had GI50 values of 0.95 µmol/L in SW620, 0.21 µmol/L in M059-Fus-1 (DNA-PK+)

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Determination of the cellular specificity of KU-0060648 for DNA-PK–dependent cell survival following exposure to etoposide or doxorubicin. A and B, clonogenic survival of V3-YAC and V3 cells exposed to etoposide alone (Etop; solid symbols) or in combination with 1 µmol/L KU-0060648 (open symbols) for 16 hours before seeding for colony formation. C and D, clonogenic survival of M059-Fus-1 and M059J cells exposed to etoposide alone (black bars) or in combination with 1 µmol/L KU-0060648 (white bars) for 16 hours before seeding for colony formation. E and F, clonogenic survival M059-Fus-1 and M059J cells exposed to doxorubicin alone (Dox; black bars) or in combination with 1 µmol/L KU-0060648 (white bars) for 16 hours before seeding for colony formation. Data are the mean ± SD of 3 independent experiments. G, M059J and M059-Fus-1 cells were exposed to 1 µmol/L KU-0060648 (K) or 0.1 µmol/L ZSTK474 (Z) for 1 hour. Cell lysates were prepared and the relative levels of PI-3K–dependent AKT Ser473 phosphorylation determined by Western blot.
LoVo, 0.27 μmol/L in MCF7, 0.41 μmol/L in T47D, and 1 μmol/L in MDA-MB-231.

In comparison with the profound growth inhibition, the cytotoxicity of KU-0060648 was much less marked. Following a 16-hour exposure to 1 μmol/L KU-0060648, overall cell survival rate was more than 80% in each of the cell lines except for MDA-MB-231 cells, which had a survival rate of 41% (Fig. 2A). KU-0060648 (1 μmol/L) markedly enhanced the cytotoxicity of doxorubicin and etoposide in all cell lines (Fig. 2B and Table 1). In SW620 cells, KU-0060648 enhanced the cytotoxicity of 1 μmol/L etoposide by more than 100-fold and enhanced the cytotoxicity of 10 nmol/L doxorubicin by more than 10-fold. However, in LoVo, MCF7, and T47D cells, the enhancement of doxorubicin cytotoxicity (50- to 100-fold) was greater than that of etoposide (< 10-fold). The lowest potentiation of etoposide or doxorubicin cytotoxicity by KU-0060648 was observed in MDA-MB-231 cells, in which a 3- to 4-fold sensitization was observed.

Plasma pharmacokinetics of KU-0060648 following different routes of administration

The plasma pharmacokinetic parameters determined after administration of 10 mg/kg KU-0060648 to Balb/C...
mice by various routes are given in Tables 2 and 3. The percentage bioavailability of KU-0060648 following oral administration was found to be 100% or more. The pharmacokinetic parameters of KU-0060648 following i.p. administration were found to be similar to that when given i.v., with 78% bioavailability.

**Tissue distribution of KU-0060648 in MCF7 and SW620 tumor-bearing mice following i.v. administration**

Following administration of KU-0060648 (12.5 mg/kg i.v.) to mice bearing either MCF7 or SW620 xenografts, KU-0060648 distributed extensively to the tumor and was retained after clearance from the plasma (Tables 2 and 3). Concentrations of KU-0060648 of more than 1 μmol/L (a level resulting in chemosensitization in vitro) were maintained in the tumor for at least 4 hours.

**DNA-PK activity determined ex vivo in SW620 tumor samples**

To investigate whether levels of KU-0060648 achieved within tumors were sufficient to have a biologic effect, we determined DNA-PK activity by measuring DNA-PK–dependent phosphorylation of a p53 peptide substrate (Ser15). Comparison of tumor samples taken from control animals or animals treated with 2.5 or 25 mg/kg i.v. KU-0060648 revealed a dose-dependent reduction of p53 (Ser15) phosphorylation. Parallel measurement of KU-0060648 PK revealed that tumor concentrations of KU-0060648, following a 25 mg/kg i.v. dose, were sufficient to give almost 100% inhibition of DNA-PK activity for at least 4 hours (Fig. 3).

**Antitumor activity of etoposide and KU-0060648 in SW620 or MCF7 tumor–bearing mice**

We investigated the antitumor activity of KU-0060648 against both MCF7 and SW620 xenografts. Mice bearing MCF7 tumor xenografts were treated with etoposide phosphate, alone and in combination with KU-0060648 (Fig. 4A). Tumors in control mice reached 4 times their starting volume (RTV4) at a median time of 10 days (mean time to RTV4 = 9.6 ± 1.9 days). Treatment with etoposide phosphate alone caused a median growth delay of 38 days (time to RTV4 = 48 days; mean time to RTV4 = 31.7 ± 8.6 days; \( P = 0.0001 \)). Treatment with KU-0060648 alone caused a median growth delay of 30 days (time to RTV4 = 40 days; mean time to RTV4 = 43.3 ± 3.6 days; \( P = 0.0467 \)), and combination of treatments caused a median growth delay of 55 days (time to RTV4 = 65 days; mean time to RTV4 = 52 ± 6.8 days; \( P = 0.006 \)). The toxicity of KU-0060648 and etoposide phosphate alone was negligible (maximum body weight loss = 3%), and the combination of drugs did not cause unacceptable toxicity (maximum body weight loss = 7%). In the SW620 xenograft model, tumors in control mice reached RTV4 at a median time of 5.5 days, and consistent with *in vitro* studies, KU-0060648 alone did not cause any tumor growth delay (\( P = 0.4573 \)). Treatment with etoposide phosphate alone caused a median tumor growth delay of 1.9 days.

**Table 1. Sensitization of human colon and breast cancer cell lines to doxorubicin and etoposide by KU-0060648**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SW620</th>
<th>LoVo</th>
<th>MCF7</th>
<th>T47D</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μmol/L Etop.</td>
<td>4.7 ± 2.0</td>
<td>3.5 ± 0.5</td>
<td>23 ± 9</td>
<td>24 ± 4</td>
<td>16 ± 10</td>
</tr>
<tr>
<td>Etop. + KU</td>
<td>0.05 ± 0.02</td>
<td>0.05 ± 0.02</td>
<td>3.1 ± 1.0</td>
<td>6.0 ± 1.7</td>
<td>4.1 ± 1.5</td>
</tr>
<tr>
<td>Fold enhancement</td>
<td>105 ± 16</td>
<td>6.6 ± 2.6</td>
<td>8.9 ± 4.9</td>
<td>4.1 ± 0.6</td>
<td>3.6 ± 1.2</td>
</tr>
<tr>
<td>100 nmol/L Dox.</td>
<td>0.06 ± 0.06</td>
<td>1.4 ± 0.9</td>
<td>42.3 ± 13</td>
<td>9.0 ± 3.2</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>100 nmol/L Dox. + KU</td>
<td>0.004 ± 0.002</td>
<td>0.03 ± 0.03</td>
<td>0.41 ± 0.08</td>
<td>0.19 ± 0.12</td>
<td>0.47 ± 0.14</td>
</tr>
<tr>
<td>Fold enhancement</td>
<td>12 ± 8</td>
<td>73 ± 29</td>
<td>107 ± 43</td>
<td>58 ± 35</td>
<td>3.8 ± 0.6</td>
</tr>
</tbody>
</table>

NOTE: Cells were exposed to etoposide (Etrop), doxorubicin (Dox), and/or KU-0060648 (KU, 1 μmol/L) for 16 hours before seeding for colony formation.

*Fold enhancement = survival cytotoxic alone/survival cytotoxic alone + KU-0060648*

**Table 2. Plasma PK parameters following administration of KU-0060648 by the i.v., p.o., or i.p route in female Balb/C mice**

<table>
<thead>
<tr>
<th>Dose and route</th>
<th>KU-0060648 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i.v.</td>
</tr>
<tr>
<td>Cmax, μg/mL</td>
<td>1.9</td>
</tr>
<tr>
<td>Tmax (min)</td>
<td>5</td>
</tr>
<tr>
<td>( AUC_{0-\infty} ), μg·h/mL</td>
<td>242</td>
</tr>
<tr>
<td>( T_{1/2} ) (min)</td>
<td>102</td>
</tr>
<tr>
<td>CL (ml/kg/min)</td>
<td>41</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>119</td>
</tr>
</tbody>
</table>

NOTE: Data are calculated from mean values obtained at 5, 10, 15, 30, 60, 90, 120, 180, 240, and 360 minutes after administration from 3 mice per time point.

Abbreviations: \( C_{max} \), concentration maximum; \( T_{max} \), time of \( C_{max} \); \( AUC_{0-\infty} \), area under the curve; \( T_{1/2} \), elimination half-life; CL, clearance.
of 1 day (time to RTV4 = 6.5 days), which was extended to 3.5 days (time to RTV4 = 9 days) by coadministration of KU-0060648 once daily. When KU-0060648 was administered twice daily, a growth delay of a further 2 days (time to RTV4 = 11 days) was observed. However, none of the tumor growth delays were statistically significant due to the rapid growth of SW620 tumors and requirements to kill mice with large tumors, resulting in a reduced sample size. Neither KU-0060648 nor etoposide phosphate alone caused any significant toxicity (no body weight loss), and the combination of drugs did not cause unacceptable toxicity (maximum body weight loss = 11%).

Discussion

Both DNA-PK and PI-3K, by virtue of their role in DSB repair and the promotion of cell proliferation, respectively, are attractive targets for the development of novel anticancer therapies. KU-0060648 is a submicromolar inhibitor of DNA-PK and PI-3K in MCF7 cells, with a 10-fold reduced potency against DNA-PK and more than 250-fold reduced potency against PI-3K in SW620 cells. The potency in intact cells was substantially lower than in the cell-free assay (Supplementary Fig. S1). As KU-0060648 is an ATP-competitive inhibitor, the discrepancy may be attributable to high intracellular ATP concentration. The approximately 10-fold difference in IC50 values for KU-0060648 between MCF7 and SW620 cells might reflect different cellular ATP concentrations between cell lines or cell-specific differences in uptake or efflux, resulting in higher concentrations within the MCF7 cells. Although, neither hypothesis has been tested experimentally, inhibition of PI-3K–dependent AKT phosphorylation by ZSTK474 (control compound) was also 10-fold less potent in SW620 cells compared with MCF7 cells, and this could also be dependent on potential differences in uptake and ATP concentrations between the 2 cell lines. In further support of this hypothesis, lower levels of KU-0060648 were observed in SW620 tumor xenografts than MCF7 tumor xenografts, although the difference was not statistically significant (Table 3). However, even accounting for a potential difference in KU-0060648 accumulation within the cells, the lack of PI-3K inhibition by KU-0060648 in SW620 cells was striking. This suggests that KU-0060648 may inhibit PI-3K in a cell-line–dependent manner. Alternatively, the complete lack of AKT phosphorylation inhibition in response to KU-0060648 compared with the dose-dependent response to ZSTK474 in the SW620 cells may reflect differential effects of the compounds on the PI-3K isoforms and their relative activities in SW620 cells. Of the 4 PI-3K isoforms, KU-0060648 has the greatest potency against PI-3Kδ and the least potency against PI-3Kγ (Supplementary Fig. S1). Conversely, ZSTK474 has the greatest potency against PI-3Kγ, and the least potency against PI-3Kδ (36).

Consistent with the well-established role of PI-3K in cell proliferation (20, 37), KU-0060648 caused substantial growth inhibition across a panel of human cancer cell lines. The degree of growth inhibition was cell line–specific. The apparent lack of PI-3K inhibition by KU-0060648 in SW620 cells might explain why a 5-day exposure to KU-0060648 (1 µmol/L) only conferred 50% growth inhibition in SW620 cells, compared with more than 95% growth inhibition in MCF7 cells. Similarly, KU-0060648 was found to have single-agent activity in mice bearing MCF7 xenografts (causing almost as much tumor growth delay as etoposide alone, at the doses studied), but had no single-agent activity in mice bearing SW620 xenografts. In addition to the differential effects on AKT phosphorylation, a further explanation for the different effects of KU-0060648 on the growth of SW620 and MCF7 xenografts is that MCF7 cells (unlike SW620) harbor a PI-3K mutation, and there is evidence that PI-3K mutations confer sensitivity to single-agent PI-3K inhibitors (38, 39). Consistent with these studies, we show that KU-0060648 conferred greater in vitro growth inhibition

### Table 3. KU-0060648 tumor and plasma concentration following a 12.5 mg/kg i.v. dose in mice bearing subcutaneous MCF7 or SW620 human xenografts

<table>
<thead>
<tr>
<th></th>
<th>MCF7</th>
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<th>SW620</th>
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<tbody>
<tr>
<td></td>
<td>Plasma, µmol/L</td>
<td>Tumor, µmol/L</td>
<td>Plasma, µmol/L</td>
<td>Tumor, µmol/L</td>
</tr>
<tr>
<td>60 min</td>
<td>1.0 ± 0.6</td>
<td>7.0 ± 3.8</td>
<td>2.1 ± 0.5</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>240 min</td>
<td>0.8 ± 0.5</td>
<td>4.7 ± 1.1</td>
<td>0.7 ± 0.2</td>
<td>2.9 ± 0.4</td>
</tr>
</tbody>
</table>

NOTE: Data are the mean ± SD of values obtained from 3 mice per time point.
in the breast cancer cell lines MCF7 and T47D, which also harbor a PIK3CA mutation, than MDA-MB-231 cells.

The differential effects of KU-0060648 on in vitro growth inhibition across the panel of human cancer cell lines, and the effect of KU-0060648 on tumor growth delay in MCF7 xenograft-bearing mice, suggest that KU-0060648 may be effective as a single-agent therapy. The sensitivity to KU-0060648 as a single-agent seems to be largely cell line–dependent. However, characterization of the molecular determinants for the sensitivity of cancer cells to KU-0060648, potentially PIK3CA mutations, may lead to the establishment of predictive biomarkers, which could be used to identify patients sensitive to KU-0060648 as a single-agent therapy.

Studies in isogenically paired DNA-PKcs–proficient and -deficient cells (that have comparable levels of PI-3K activity, which was similarly inhibited by KU-0060648) confirm that KU-0060648 potentiation of etoposide and doxorubicin cytotoxicity in these models is largely due to DNA-PK inhibition. Substantial enhancement of etoposide and doxorubicin cytotoxicity was also observed across a panel of human cancer cell lines. However, there was significant cell line variation in the degree of enhancement. MDA-MB-231 cells were relatively resistant to sensitization to both drugs (4-fold enhancement of toxicity). In MCF7 cells, KU-0060648 increased doxorubicin cytotoxicity more than 100-fold but only increased etoposide cytotoxicity 9-fold. Conversely, KU-0060648 increased doxorubicin cytotoxicity by 12-fold and etoposide cytotoxicity more than 100-fold in SW620 cells. The differential chemosensitization of 2 similar-acting drugs by KU-0060648 do not seem to be related to the p53-status of the cells (MCF7 and LoVo have wild-type p53; refs. 40, 41, and SW620 MDA-MB-231 and T47D have mutant p53; refs. 42–44), but may depend on other molecular determinants in the cell panel that are yet to be determined.

KU-0060648 had significant single-agent antitumor activity that was comparable with that of etoposide in mice bearing MCF7 tumor xenografts, and the combination was more active than either drug alone. Neither KU-0060648 nor etoposide had any discernible effect on the growth of SW620 xenografts, but there was a modest trend toward increased antitumor activity by the combination of the 2 drugs, particularly when KU-0060648 was administered twice.

Overall, these studies show that KU-0060648 is a potent dual inhibitor of DNA-PK and PI-3K, which inhibits cell growth and enhances the cytotoxicity of topoisomerase II poisons in a cell line–dependent manner. KU-0060648 showed good oral bioavailability and pharmacokinetics, resulting in concentrations within the tumor that are commensurate with cellular DNA-PK and PI-3K inhibition, in vitro cell growth inhibition, and in vitro chemosensitization. Furthermore, KU-0060648 conferred complete inhibition of DNA-PK activity in SW620 tumors. The differential sensitivities of SW620 and MCF7 xenografts suggest that there may be a tumor-specific response to KU-0060648, which highlights the need to identify predictive biomarkers, such as PI-3K mutation, for the selection of patients that are most likely to respond to KU-0060648. These data provide excellent proof-of-principle evidence that improvements in the antitumor activity of doxorubicin and etoposide are achievable through the dual inhibition of PI-3K and DNA-PK.

Disclosure of Potential Conflicts of Interest

N.J. Curtin, R.J. Griffin, and D.R. Newell have received research funding from KuDOS/Astrazeneca. K. Meneer, H. Jenkins, C.J. Richardson,
N.M.B. Martin, A. Slade, and J. Bardos were former employees of KuDOS Pharmaceuticals. P. Thommes and G.C.M. Smith are currently employed by AstraZeneca. R.J. Griffin and N.J. Curtin have received commercial research grants from AstraZeneca Pharmaceuticals. D.R. Newell has honoraria from speaker’s bureau from Leeds University and is a consultant/advisory board member of Astex Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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References


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