Context-Dependent Antagonism between Akt Inhibitors and Topoisomerase Poisons

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ABSTRACT

Signaling through the phosphatidylinositol-3 kinase (PI3K)/Akt pathway, which is aberrantly activated in >50% of carcinomas, inhibits apoptosis and contributes to drug resistance. Accordingly, several Akt inhibitors are currently undergoing preclinical or early clinical testing. To examine the effect of Akt inhibition on the activity of multiple widely used classes of antineoplastic agents, human cancer cell lines were treated with the Akt inhibitor A-443654 [(2S)-1-(1*H*-indol-3-yl)-3-[5-(3-methyl-2*H*-indazol-5-yl)pyridin-3-yl]oxypropan-2-amine; ATP-competitive] or MK-2206 (8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-2*H*-[1,2,4]triazolo[3,4-*f*][1,6]naphthyridin-3-one; dihydrochloride; allosteric inhibitor) or with small interfering RNA (siRNA) targeting phosphoinositide-dependent kinase 1 (PDK1) along with cisplatin, melphalan, camptothecin, or etoposide and assayed for colony formation. Surprisingly different results were observed when Akt inhibitors were combined with different drugs. Synergistic effects were observed in multiple

cell lines independent of PI3K pathway status when A-443654 or MK-2206 was combined with the DNA cross-linking agents cisplatin or melphalan. In contrast, effects of the Akt inhibitors in combination with camptothecin or etoposide were more complicated. In HCT116 and DLD1 cells, which harbor activating *PI3KCA* mutations, A-443654 over a broad concentration range enhanced the effects of camptothecin or etoposide. In contrast, in cell lines lacking activating *PI3KCA* mutations, partial inhibition of Akt signaling synergized with camptothecin or etoposide, but higher A-443654 or MK-2206 concentrations (>80% inhibition of Akt signaling) or PDK1 siRNA antagonized the topoisomerase poisons by diminishing DNA synthesis, a process that contributes to effective DNA damage and killing by these agents. These results indicate that the effects of combining inhibitors of the PI3K/Akt pathway with certain classes of chemotherapeutic agents might be more complicated than previously recognized.

Introduction

The phosphatidylinositol-3 kinase (PI3K)/Akt pathway has become an important target of new anticancer agents (Engelman, 2009; Pal et al., 2010; Sheppard et al., 2012; Slomovitz and Coleman, 2012; Rodon et al., 2013). Signaling through this pathway involves the sequential action of the lipid kinase PI3K, which produces PIP₃ (phosphatidylinositol-3,4,5-trisphosphate); PIP₃-mediated activation of the serine/threonine kinase phosphoinositide-dependent kinase 1 (PDK1); and PIP₃-mediated recruitment of Akt isoforms to the plasma membrane, where they are activated by PDK1-catalyzed phosphorylation (Bjornsti and Houghton, 2004; Manning and Cantley, 2007; Fayard et al., 2010). A variety of genetic and epigenetic changes, including activating mutations in growth factor receptors or *PI3K*,

amplification of *AKT2*, or loss of phosphatase and tensin homolog, which ordinarily dephosphorylates PIP₃ to extinguish the signal, lead to sustained activation of Akt in >50% of human cancers, including carcinomas of the ovary, breast, prostate, pancreas, cervix, colon, endometrium, and lung, as well as lymphoid and myeloid leukemias (Bjornsti and Houghton, 2004; Manning and Cantley, 2007; Engelman, 2009; Fayard et al., 2010; Vasudevan and Garraway, 2010; Slomovitz and Coleman, 2012).

Once activated, Akt enhances cell motility, proliferation, and survival (Manning and Cantley, 2007; Vasudevan and Garraway, 2010) by phosphorylating a number of protein substrates, including a subset of the Foxo transcription factors; the cyclin-dependent kinase inhibitors p21^{Waf1/Cip1} and p27^{Kip1}; the apoptotic regulator X-linked inhibitor of apoptosis protein; the GTPase activating protein tuberous sclerosis protein 1, which regulates protein synthesis through the mammalian target of the rapamycin pathway; and glycogen synthase kinase-3 β (GSK-3 β), which controls not only glycogen synthesis, but also

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ABBREVIATIONS: A-443654, (2S)-1-(1*H*-indol-3-yl)-3-[5-(3-methyl-2*H*-indazol-5-yl)pyridin-3-yl]oxypropan-2-amine; BrdU, 5-bromo-2'-deoxyuridine; CI, combination index; GSK-3 β , glycogen synthase kinase-3 β ; IC₅₀, concentration that inhibits colony formation by 50%; MK-2206, 8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-2*H*-[1,2,4]triazolo[3,4-*f*][1,6]naphthyridin-3-one; dihydrochloride; PBS, phosphate-buffered saline; PDK1, phosphoinositide-dependent kinase 1; PI, propidium iodide; PI3K, phosphatidylinositol-3 kinase; PIP₃, phosphatidylinositol-3,4,5-trisphosphate; siRNA, small interfering RNA.

cell survival through regulation of nuclear factor κ B-mediated transcription and Mcl-1 stability (Manning and Cantley, 2007; Vasudevan and Garraway, 2010). Collectively, Akt-mediated phosphorylation of these polypeptides leads to enhanced proliferation of tumor cells as well as their increased survival after chemotherapy and radiation in vitro and in vivo.

The realization that PI3K/Akt pathway activation is widespread and portends a poor response to existing therapies (Vivanco and Sawyers, 2002; Bussink et al., 2008; Yuan and Cantley, 2008) has led to current attempts to develop a variety of PI3K and Akt inhibitors as potential anticancer drugs (Engelman, 2009; Pal et al., 2010; Sheppard et al., 2012; Slomovitz and Coleman, 2012; Rodon et al., 2013). One such molecule is A-443654 [(2*S*)-1-(1*H*-indol-3-yl)-3-[5-(3-methyl-2*H*-indazol-5-yl)pyridin-3-yl]oxypropan-2-amine], an indazole-pyridine-based inhibitor that selectively and reversibly binds to the ATP sites of Akt1, Akt2, and Akt3 (Luo et al., 2005; Shi et al., 2005) to diminish phosphorylation of Akt downstream targets with an IC_{50} of 100–1000 nM in intact cells (Luo et al., 2005; Shi et al., 2005). Additional studies have demonstrated that A-443654 increases the apoptosis induced by camptothecin, doxorubicin, paclitaxel, or etoposide in the H460 lung cancer cell line and T cell ALL cells (Shi et al., 2005; Fala et al., 2008). MK-2206 (8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-2*H*-[1,2,4]triazolo[3,4-*f*][1,6]naphthyridin-3-one; dihydrochloride), an allosteric inhibitor of Akt1, Akt2, and Akt3, likewise is reported to synergize with camptothecin, doxorubicin, gemcitabine, 5-fluorouracil, and carboplatin in the H460 lung and A2780 ovarian cancer lines as assessed by CellTiter-Glo assay in vitro (Hirai et al., 2010). It has also been observed that cells with certain activating mutations above Akt in the PI3K/Akt pathway are hypersensitive to MK-2206 (She et al., 2008; Hanrahan et al., 2012). Whether the same principle applies to the use of Akt inhibitors in combination therapy is less clear.

In the present study, we examined the effect of combining A-443654 or MK-2206 with several different classes of anticancer agents, including topoisomerase poisons and DNA cross-linking agents, in solid tumor cell lines with or without PI3K mutations. Results of these experiments demonstrate that antiproliferative effects of cisplatin and melphalan are enhanced independent of *PI3KCA* mutation status by several treatments that inhibit Akt signaling. In contrast, effects of combining Akt inhibitors with the prototypic topoisomerase poisons camptothecin and etoposide were more complicated, with synergy observed in cells harboring activating *PI3KCA* mutations but lack of synergy, particularly at high Akt inhibitor concentrations, in cells with wild-type *PI3KCA*. These observations highlight the potential importance of cellular context in determining whether Akt inhibitors synergize with certain types of DNA-damaging agents.

Materials and Methods

A-443654 was kindly provided by Saul Roseman and Vincent Giranda (Abbott Laboratories, Abbott Park, IL). Additional reagents were purchased from the following suppliers: MK-2206 from ChemieTek (Indianapolis, IN); cisplatin, melphalan, etoposide, camptothecin, and propidium iodide (PI) from Sigma-Aldrich (St. Louis, MO); and Opti-MEM medium and Lipofectamine 2000 from Invitrogen (Carlsbad, CA). Antibodies to the following antigens were purchased as follows: PDK1, phospho-Ser⁴³-Akt, Akt, phospho-Ser³-GSK-3 β ,

phospho-Thr²⁴⁶-Proline-rich Akt substrate of 40 kDa (PRAS40), PRAS40, and c-Raf from Cell Signaling Technology (Beverly, MA); BrdU (5-bromo-2'-deoxyuridine) from Becton-Dickinson (Mountain View, CA); phospho-Ser¹³⁹-histone H2AX from Active Motif (Carlsbad, CA); and GSK-3 β from Oncogene Research (Cambridge, MA). Monoclonal antibodies raised against topoisomerase I, topoisomerase II α , lamin A, and heat shock protein 90 β were kind gifts from Y.-C. Cheng (Yale University, New Haven, CT), Udo Kellner (Otto-von-Guericke University, Magdeburg, Germany), Frank McKeon (Harvard Medical School, Boston, MA), and David Toft (Mayo Clinic, Rochester, MN), respectively.

Cell Culture. All media contained 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 μ g/ml streptomycin, and 2 mM glutamine. A549 human lung adenocarcinoma (from American Type Culture Collection, Rockville, MD) and HeLa cervical carcinoma cells were cultured in RPMI 1640 (medium A). T98G human glioblastoma cells (C. David James, University of California, San Francisco, CA) were cultured in minimal essential medium supplemented with 1 mM sodium pyruvate. MDA-MB-231 human breast adenocarcinoma cells were cultured in minimal essential medium containing Earle's salts supplemented with 10 mM HEPES, 10 μ g/ml insulin, 1 mM sodium pyruvate, and essential and nonessential amino acids. HCT116 cells and their derivatives containing only the wild-type or mutant *PI3KCA* alleles (Samuels et al., 2005) as well as DLD1 colorectal cancer cells (Johns Hopkins University Genetic Resources Core Facility Cell Center, Baltimore, MD) were cultured in McCoy's 5A medium. All lines except MDA-MB-231 and HeLa were derived from males.

After subconfluent monolayers were trypsinized, aliquots containing 500 A549 cells were plated in multiple 35-mm dishes containing 2 ml of medium A and incubated for 12–16 hours at 37°C to allow cells to attach. Serial dilutions of drugs or equivalent volumes of diluent were then added to triplicate plates. After a 24-hour incubation, plates were washed twice in serum-free RPMI 1640 and incubated in drug-free medium A for an additional 7 days. The resulting colonies were stained with Coomassie Blue and counted. Diluent-treated control plates typically contained 150–200 colonies. Colony-forming assays in other lines were performed similarly except that 250 (T98G, HeLa, DLD1) or 500 (MDA-MB-231, HCT116, and derivatives) cells were plated and treatments were performed in the media indicated previously.

Analysis of Combined Drug Effects. Concentration-effect curves were initially generated for each agent to estimate its IC_{50} for the cell line under study. In subsequent experiments, cells were treated with serial dilutions of each drug individually and with both drugs simultaneously at concentrations that typically corresponded to 3/8, 1/2, 3/4, 1, and 1-1/2 times the camptothecin, etoposide, cisplatin, or melphalan IC_{50} in the presence of three to six fixed A-443654 or MK-2206 concentrations. Fractional survival (*f*) was calculated by dividing the number of colonies in drug-treated plates by the number of colonies in control plates. Data were subsequently analyzed as described (Chou and Talalay, 1984). In brief, $\log[(1/f) - 1]$ was plotted against $\log(\text{drug dose})$. From the resulting median effect lines, the *x*-intercept ($\log IC_{50}$) and slope *m* (a measure of sigmoidicity) were calculated for each drug and for the combination by the method of least squares. These parameters were then used to calculate the combination index (CI) according to the assumption that the effects of the agents are mutually exclusive (Chou and Talalay, 1984). In this method, which is equivalent to isobologram analysis (Berenbaum, 1989), synergy is indicated by $CI < 1$, additivity by $CI = 1$, and antagonism by $CI > 1$.

Unless otherwise indicated, drug treatments were repeated until at least three independent experiments yielded correlation coefficients $R > 0.9$ for all three median effect lines. The CI was then plotted as a function of the fraction of cells affected ($1 - f$) or as a function of drug concentrations as indicated in the individual figures. Bar graphs were used to summarize multiple independent experiments ($n = 3$ unless otherwise stated) by showing the mean and S.D. of colony formation after the indicated treatments.

Small Interfering RNA Transfections. On day 1, A549 cells (8×10^5) were plated in 35-mm tissue culture dishes and incubated

overnight. On day 2, after cells were washed twice with Opti-MEM medium, 2 ml of Opti-MEM was added to each plate. Then 400 nmol of luciferase small interfering RNA (siRNA) (Dharmacon, Lafayette, CO) or PDK1 siRNA (Zhao et al., 2002) were complexed with 10 μ l of Lipofectamine 2000 in 0.5 ml of Opti-MEM for 20 minutes. Following addition of the lipid-siRNA complexes, cultures were incubated for 4–6 hours before addition of 1 ml Opti-MEM containing 35% (v/v) fetal bovine serum. The transfections were repeated on day 3. On day 4, the cultures were trypsinized and replated in 100-mm tissue culture dishes containing medium A. On day 5, cells were harvested for immunoblotting, cell cycle analysis, and BrdU incorporation, or exposed to drugs for colony-forming assays as described earlier.

BrdU Incorporation into DNA. Following treatment with A-443654 for 12 or 24 hours, cells were incubated with 20 μ M BrdU for 30 minutes, trypsinized, centrifuged at 200g, washed in ice-cold Dulbecco's calcium- and magnesium-free phosphate-buffered saline (PBS), and fixed in 66% (v/v) ethanol at -20°C . After rehydration, samples were incubated with 0.04% (w/v) pepsin, treated with 2 N HCl, neutralized, stained with anti-BrdU antibody and fluorescein-conjugated secondary antibody followed by PI, and subjected to flow cytometry as previously described (Galvez-Peralta et al., 2008). After collection of 20,000 events per sample, BrdU incorporation and cell cycle distribution were analyzed using Becton Dickinson (San Jose, CA) CellQuest software. Relative levels of BrdU incorporation were calculated as the ratio of the mean fluorescence intensity of drug-treated samples to mean fluorescence intensity of diluent-treated samples.

Phospho-Ser¹³⁹-Histone H2AX Staining. Following treatment of A549 cells with diluent, 250 nM A-443654, or 1000 nM MK-2206 for 18 hours, diluent or camptothecin (final concentration 40 nM) was added and the incubation was continued for an additional 6 hours. At the completion of the incubation, cells were fixed for 15 minutes on ice with 4% (w/v) paraformaldehyde in PBS, permeabilized with 0.25% (w/v) Triton X-100 in PBS for 15 minutes on ice followed by 0.1% (w/v) sodium dodecyl sulfate in PBS for 5 minutes at 21°C , washed four times with 0.1% (w/v) Triton + 0.1% (w/v) bovine serum albumin in PBS followed by three times with PBS, and blocked in 10% (w/v) powdered nonfat milk in PBS. Slides were stained with anti-phospho-Ser¹³⁹-histone H2AX followed by Alexa Fluor 568-conjugated anti-rabbit IgG using incubation and wash conditions previously described for other antigens (Adjei et al., 2000). After incubation with 1 μ g/ml Hoechst 33258 to visualize nuclei, cells were mounted in Slow-Fade Gold antifade reagent (Invitrogen) and subsequently examined on a Zeiss Axioplan microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a Zeiss 100 $\times$ numerical aperture 1.40 Plan-Apochromat lens. Images were captured using a Zeiss AxioCam MRM and Zeiss Zen 2012 Blue Edition software.

Immunoblotting. After treatment with drug or diluent as indicated in the individual figures, cells were washed three times with ice-cold RPMI 1640 medium containing 10 mM HEPES (pH 7.4 at 4°C) and solubilized by addition of 6 M guanidine hydrochloride containing 250 mM Tris-HCl (pH 8.5 at 20°C), 10 mM EDTA, 1% (v/v) 2-mercaptoethanol, and 1 mM freshly added phenylmethylsulfonyl fluoride. After samples were prepared for electrophoresis as previously described (Kaufmann et al., 1997), aliquots containing 50 μ g of protein [determined by the bicinchoninic acid method (Smith et al., 1985)] were separated on SDS-polyacrylamide gels containing 5–15% (w/v) acrylamide gradients, electrophoretically transferred to nitrocellulose, and probed with immunologic reagents as reported (Kaufmann, 2001). After chemiluminescent detection, X-ray films were scanned on an Epson model 4870 scanner (Epson America, Long Beach, CA) and quantitated using ImageJ 1.47 (ImageJ64; NIH, Bethesda, MD) (Schneider et al., 2012). Data were expressed as the ratio of the signal for phospho-Ser⁹-GSK-3 β to the signal for total GSK-3 β in each drug-treated sample and normalized to the same ratio in diluent-treated cells from the same experiment. Results shown are the mean $\pm$ S.D. from three independent drug exposures except for HCT116 gene targeted cells, which are the mean and range

from two separate experiments. Alternatively, cell lysates were prepared from siRNA-transfected cells and probed by immunoblotting as previously described (Arlander et al., 2003).

Results

A-443654 Inhibits Akt-Induced Phosphorylation.

Based on previous studies implicating Akt in resistance to chemotherapeutic drugs, we examined the effect of combining the Akt inhibitor A-443654 (Fig. 1A) with a variety of chemotherapeutic agents. These studies initially used A549 (*KRAS* mutated), T98G (*INK4A* deleted, *TP53* mutated), and MDA-MB-231 cells (*KRAS* mutated). Studies in these cell lines demonstrated that A-443654 caused decreased phosphorylation of the Akt substrates GSK-3 α and GSK-3 β on Ser²¹ and Ser⁹, respectively (Fig. 1, B–G). Further experiments demonstrated that A-443654 modestly inhibited colony formation when applied at concentrations $\leq$ 250 nM for 24 hours, although larger effects were observed at higher concentrations (Fig. 1, H–J).

A-443654 Synergizes In Vitro with DNA Cross-Linking Agents but Has More Complicated Effects in Combination with Topoisomerase Poisons. To determine whether Akt inhibition enhances the antiproliferative effects of DNA cross-linking agents, A549 cells were treated with increasing concentrations of cisplatin or melphalan in the absence or presence of A-443654 for 24 hours, washed, and allowed to form colonies. As indicated in Fig. 2, A and B, A-443654 at 62.5–250 nM increased the effects of cisplatin on colony formation. Analysis by the median effect method (Fig. 2C) demonstrated that the CI was below 1.0 over a wide range of drug concentrations, indicating synergy. Likewise, A-443654 enhanced the antiproliferative effects of melphalan (Fig. 2, D and E) in a synergistic fashion (Fig. 2F).

Further experiments examined the effects of Akt inhibition in combination with topoisomerase I and II poisons. Because A-443654 alters the uptake of topotecan (M. Gálvez-Peralta and S. H. Kaufmann, unpublished observations), these studies used camptothecin as a prototypic topoisomerase I-directed agent. When combined with camptothecin, the effects of the Akt inhibitor were highly dose-dependent. In particular, 62.5 or 125 nM A-443654 enhanced the antiproliferative effect of camptothecin in A549 cells (Fig. 3A, $\blacktriangledown$; data not shown). In contrast, even though 250 nM A-443654 inhibited colony formation by itself, addition of camptothecin produced much less effect on colony formation (Fig. 3A, $\square$, and B). Plotting the CI as a function of the percentage of cells affected (Chou and Talalay, 1984) yielded a confusing and inconsistent picture (Fig. 3C). However, plots of CI as a function of drug concentration indicated that the CI was <1 with 62.5 or 125 nM A-443654 but generally >1 with 250 nM A-443654 (Fig. 3D).

To rule out the possibility that these effects were unique to A-443654, we tested the allosteric Akt inhibitor MK-2206, which is structurally (Fig. 1A) and mechanistically distinct from A-443654 (Hirai et al., 2010). MK-2206-induced inhibition of GSK-3 β phosphorylation was maximal at 125–250 nM (Fig. 3E, upper panel), whereas inhibition of phosphorylation of the Akt substrate PRAS40 occurred progressively up to 2000 nM (Fig. 3E, third panel). These results, which indicate that more MK-2206 is required to inhibit phosphorylation of some Akt substrates compared with others, are reminiscent of differential

Fig. 1. Effects of A-443654 on polypeptide levels, substrate phosphorylation, and clonogenic survival. (A) Chemical structure of the Akt inhibitors A-443654 and MK-2206. (B–D) After A549 (B), T98G (C), or MDA-MB-231 cells (D) were treated for 24 hours with the indicated concentration of A-443654, whole-cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with antibodies that recognize the indicated antigens. Numbers at left are molecular mass markers in kDa. (E–G) The relative signal for phospho-Ser⁹-GSK-3 β in A-443654-treated A549 (E), T98G (F), and MD-MB-231 cells (G) was quantitated as described in *Materials and Methods*. Error bars represent the mean $\pm$ S.D. of three independent determinations. (H–J) Representative clonogenic experiments showing the effects of a 24-hour exposure of A549 (H), T98G (I), or MDA-MB-231 (J) to A-443654. Error bars represent the mean $\pm$ 1 S.D. of triplicate aliquots.

efforts of the allosteric inhibitor rapamycin on some mammalian target of rapamycin substrates versus others (Choo and Blenis, 2009). Like A-443654, MK-2206 over this concentration range enhanced the antiproliferative effects of cisplatin and melphalan (Fig. 3F; data not shown). In contrast, MK-2206 concentrations of 500 nM and above diminished rather than enhanced the antiproliferative effects of camptothecin in A549 cells (Fig. 3, G and H), resulting in CI values >1 (Fig. 3I). Accordingly, the antagonism was not unique to A-443654 or to inhibition by a catalytic Akt inhibitor.

When A-443654 was combined with the topoisomerase II poison etoposide, similar results were observed. At low A-443654 concentrations that failed to inhibit Akt-mediated phosphorylation completely, the antiproliferative effects of etoposide were increased (Fig. 3J), whereas at higher A-443654 concentrations, the effects of the combination were similar to those of etoposide alone (Fig. 3, J and K). Mathematical analysis indicated synergy (CI <1) at 62.5 and 125 nM A-443654 and antagonism (CI >1) at 250–500 nM A-443654, which was again more evident when CI was plotted

Fig. 2. Synergistic effects of A-443654 on cisplatin and melphalan sensitivity. (A and D) A549 cells were treated for 24 hours with the indicated concentration of cisplatin (A) or melphalan (D) in the presence of diluent (●) or A-443654 at 62.5 nM (▽) or 250 nM (□). In these experiments, 125 nM A-443654 had intermediate effects that are not shown to simplify the graphs. At the completion of the incubation, cells were washed and incubated for 7 days in drug-free medium. Error bars represent the mean $\pm$ S.D. of triplicate aliquots. (B and E) Summarized results of three independent experiments showing colony formation after treatment with A-443654 without or with cisplatin (B) or melphalan (E). Error bars in these and subsequent bar graphs indicate $\pm$ 1 S.D., with *P* values showing results of paired *t* tests. (C and F) CI values calculated from data in A and D, respectively, under the assumption that effects of the agents are mutually exclusive. Results are representative of three independent experiments. DMSO, dimethylsulfoxide.

as a function of drug concentrations rather than a fraction of cells affected (Fig. 3, L and M).

To rule out the possibility that these results were unique to A549 cells, we also examined the combination of A-443654 and camptothecin in MDA-MB-231 and T98G cells. As was the case in A549 cells, low A-443654 concentrations enhanced the effects of camptothecin slightly (Fig. 4A), whereas 250 nM A-443654, which inhibited Akt-mediated phosphorylation by 70% (Fig. 1G), failed to increase the impact of camptothecin alone (Fig. 4, A and B), resulting in antagonism (Fig. 4C). Similar results were observed in T98G cells (Fig. 4, D and E), where the resulting CI was, for example, 1.55 ± 0.21 at 20 nM camptothecin and 300 nM A-443654.

Because antagonism between specific PI3K/Akt pathway inhibitors and topoisomerase poisons has not been previously reported, further experiments were performed to assess its mechanistic basis.

A-443654 Diminishes Topoisomerase Levels and DNA Synthesis. Efforts to understand the observed effects focused initially on factors that contribute to the cytotoxicity

of topoisomerase I poisons, including drug accumulation, topoisomerase I levels, and the rate of ongoing DNA synthesis that converts covalent topoisomerase I–DNA complexes into cytotoxic lesions. To evaluate camptothecin accumulation, we used a flow cytometry–based assay (Fig. 5) that exploits the intrinsic fluorescence of the camptothecin (Patel et al., 2012). Cellular uptake was linear over the 1.25–10 μ M camptothecin range (Fig. 5, A and B), providing attomole sensitivity in single cells. This assay failed to provide any evidence that A-443654 was diminishing camptothecin accumulation (Fig. 5, C and D).

After treatment with the 250–300 nM A-443654 concentrations that diminished camptothecin effects in clonogenic assays, immunoblotting (Fig. 1, B–D) showed only a modest decrease in topoisomerase I that appeared to be too small to fully account for the observed antagonism. Because topoisomerase I poisons and, to a lesser extent, topoisomerase II poisons require active proliferation to be toxic (Holm et al., 1989; Hsiang et al., 1989; D'Arpa et al., 1990; Kaufmann, 1991), we evaluated the possibility that effects of A-443654 on DNA

Fig. 3. High concentrations of A-443654 or MK-2206 diminish the impact of topoisomerase I and topoisomerase II poisons. (A) A549 cells were treated for 24 hours with the indicated concentration of camptothecin in the presence of diluent (●), 125 nM A-443654 (▼), or 250 nM A-443654 (□). At the completion of the incubation, cells were washed and incubated for 7 days in drug-free medium. Data were graphed as the ratio of the number of colonies in drug-treated versus diluent-treated plates (A). In these experiments, 62.5 nM A-443654 had intermediate effects that are not shown to simplify the graphs. Error bars represent the mean $\pm$ 1 S.D. of triplicate aliquots. (B) Summarized results of five independent experiments. (C and D) CI values were calculated from data in the left panel and additional data points obtained with 62.5 nM A-443654 in the same experiment under the assumption that effects of the agents are mutually exclusive. These CIs are plotted as a function of the fraction of cells affected (C) or drug concentrations (D). (E) Cells were treated for 24 hours with the indicated concentration of MK-2206 and subjected to immunoblotting for the indicated antigens. (F) Summarized results of four independent experiments in which A549 cells were treated for 24 hours with cisplatin $\pm$ MK-2206. (G) A549 cells were treated for 24 hours with the indicated concentration of camptothecin in the presence of diluent (○), 500 nM MK-2206 (●), 1000 nM MK-2206 (▼), or 2000 nM MK-2206 (▼). At the completion of the incubation, cells were washed and incubated for 7 days in drug-free medium. (H) Summarized results from five independent experiments. (I) Using data from G, CI values were calculated and plotted. (J) A549 cells were treated for 24 hours with the indicated concentration of etoposide in the presence of diluent (●), 125 nM A-443654 (▼), or 500 nM A-443654 (■). At the completion of the incubation, cells were washed and incubated for 7 days in drug-free medium. In these experiments, 62.5 nM A-443654 had an effect similar to 125 nM A-443654, and 250 nM A-443654 had intermediate effects that are not shown in J to simplify the graph. (K) Summarized results from three independent experiments. (L and M) Using data from J, as well as results obtained with 62.5 and 250 nM A-443654 in the same experiment, CI values were calculated and plotted in L and M as described for C and D, respectively. CPT, camptothecin; DMSO, dimethylsulfoxide.

synthesis might contribute to the observed antagonism. As illustrated in Fig. 6A and summarized in Fig. 6B, A-443654 diminished BrdU incorporation into DNA of S-phase cells in a dose-dependent manner, providing another change that could diminish the effects of camptothecin and, to a lesser extent, etoposide (Holm et al., 1989; Hsiang et al., 1989; D'Arpa et al., 1990; Kaufmann, 1991). Consistent with previous reports showing that ongoing DNA synthesis is required to convert reversible cleavage complexes into cytotoxic DNA double-strand breaks (op. cit.; see also Pommier, 2006), we observed that treatment with A-443654 also diminished the

number of nuclei with camptothecin-induced phospho-Ser¹³⁹-H2AX foci (Fig. 6, C and D), a well established marker of DNA damage (Bonner et al., 2008). Similar effects were observed when cells were treated with MK-2206 (Fig. 6D). Thus, reduction in DNA synthesis during Akt inhibition was accompanied by a decrease in DNA damage induced by the topoisomerase I poison.

Comparison of Akt Inhibitors and PDK1 siRNA. To assess whether antagonism of topoisomerase I poisons might be an intrinsic feature of Akt inhibition as opposed to a unique feature of A-443654 and MK-2206, A549 cells were transfected

Fig. 4. Effects of A-443654 on camptothecin sensitivity in MDA-MB-231 and T98G cells. (A) MDA-MB-231 cells were treated for 24 hours with the indicated concentrations of camptothecin (CPT) in the presence of diluent (●), 62.5 nM (▽), or 250 nM A-443654 (□). At the completion of the incubation, cells were washed and incubated for 7 days in drug-free medium. A-443654 at 125 nM had intermediate effects that are not shown to simplify the graph. Error bars represent the mean $\pm$ 1 S.D. of triplicate aliquots from one of three independent experiments. (B) Bar graph summarizing three independent experiments. (C) CI values calculated from results shown in A as well as additional data points are graphed as a function of drug concentrations. (D) T98G cells were treated for 24 hours with the indicated concentration of camptothecin in the presence of diluent (●), 37.5 nM (▽), or 300 nM A-443654 (□), washed, and allowed to form colonies. In these experiments, 75 and 150 nM A-443654 had intermediate effects that are not shown to simplify the graph. (E) CI values calculated from data in D. DMSO, dimethylsulfoxide.

with PDK1 siRNA. As indicated in Fig. 7A, this oligonucleotide diminished phosphorylation of Akt as well as its downstream target GSK-3 β . This was accompanied by diminished BrdU incorporation into DNA (Fig. 7, B and C). When cells transfected with PDK1 siRNA were examined by clonogenic assays, PDK1 siRNA enhanced the antiproliferative effects of melphalan and cisplatin (Fig. 7, D–F). In contrast, PDK1 siRNA not only failed to sensitize cells to topoisomerase poisons, but actually diminished the antiproliferative effects of high concentrations of camptothecin (Fig. 7, G and H) and etoposide (Fig. 7, I and J) just as 250–500 nM A-443654 or MK-2206 did (Figs. 3 and 4). Similar antagonism between PDK1 siRNA and topoisomerase poisons was also observed in HeLa cells (Fig. 7, K–M).

Context-Dependent Effect of A-443654 When Combined with Topoisomerase Inhibitors. Earlier results showed that Akt inhibitors are particularly active in cells with activating *PI3KCA* mutations (Samuels et al., 2005; She et al., 2008; Hanrahan et al., 2012; Slomovitz and Coleman, 2012), reflecting the phenomenon of “oncogene addiction” in which

pathways affected by activating mutations often become the dominant pathways driving cell proliferation and survival (Sharma and Settleman, 2007; McCormick, 2011; Yap and Workman, 2012). To assess the possibility that this oncogene addiction might also impact survival after treatment with topoisomerase poisons, we also examined the effect of combining A-443654 with topoisomerase poisons in HCT116 cells, which contain an activating *PI3KCA* mutation (Samuels et al., 2005). As observed in other cell lines, A-443654 caused decreased Akt-mediated phosphorylation of GSK-3 β (Fig. 8, A and B). The HCT116 cell line was much more sensitive to the antiproliferative effects of A-443654 administered by itself (Fig. 8C), in accord with other results showing enhanced effects of Akt inhibitors in some *PI3KCA*-mutant cells (She et al., 2008; Hanrahan et al., 2012). Nonetheless, A-443654 substantially increased the effects of camptothecin on colony formation (Fig. 8, D and E). Analysis by the median effect method (Fig. 8F) demonstrated that the CI was consistently <1.0 at concentrations that inhibited colony formation by 80% or more,

Fig. 5. Effect of A-443654 on camptothecin accumulation in A549 cells. (A) Log phase A549 cells were treated for 20 minutes with the indicated concentration of camptothecin, then subjected to flow cytometry on a Becton Dickinson LSRII flow cytometer using an excitation wavelength of 355 nm and a 450 ± 25 nm bandpass filter. After collection of 20,000 events, data were analyzed using Becton Dickinson CellQuest software. (B) Graphical summary of data from A. (C) Log phase A549 cells were treated with diluent (–) or 5 μ M camptothecin (+) in the absence (–) or presence (+) of 0.5 μ M A-443654. All samples contained 0.2% dimethylsulfoxide. (D) Graphical summary of data from C and two additional independent experiments. After subtraction of mean fluorescence of samples treated with diluent or A-443654 alone, results in each experiment were expressed as the ratio of the mean fluorescence intensity due to 5 μ M camptothecin in A-443654– versus diluent-treated samples. CPT, camptothecin.

Fig. 6. Effect of A-443654 on BrdU incorporation into DNA and camptothecin-induced DNA damage. (A) A549 cells treated for 12 or 24 hours with A-443654 at the indicated concentration were pulsed for 30 minutes with 10 μ M BrdU in the continued presence of A-443654, fixed, and stained with anti-BrdU antibody and PI prior to flow microfluorimetry as described in *Materials and Methods*. (B) Effect of varying A-443654 concentrations on BrdU incorporation into S-phase cells based on flow microfluorimetry as illustrated in A. Error bars represent the mean $\pm$ S.D. from five independent experiments. (C and D) After treatment with diluent, 250 nM A-443654, or 1000 nM MK-2206 for 18 hours, camptothecin (CPT) was added to a final concentration of 40 nM, and the incubation was continued for an additional 6 hours. At the completion of the incubation, cells were fixed and stained with anti-phospho-Ser¹³⁹-histone H2AX. Representative images (C) and summarized results (D) are shown. Error bars represent the mean $\pm$ S.E.M. from four independent experiments. * $P = 0.03$; ** $P = 0.06$ relative to sample treated with camptothecin in the absence of Akt inhibitor as assessed by analysis of variance with post-hoc t tests and Bonferroni correction.

indicating synergy. Likewise, A-443654 enhanced the antiproliferative effects of etoposide in HCT116 cells (Figs. 8, G–I). Examination of DLD1 cells, which also contain an activating *PI3KCA* mutation (Samuels et al., 2005), demonstrated that A-443654 enhanced the antiproliferative effects of etoposide (Fig. 8, J and K) and camptothecin (Fig. 8L) in this cell line as well. These observations raised the possibility that Akt inhibition might synergize with topoisomerase poisons specifically in cell lines with *PI3KCA* mutations.

To further examine this possibility, gene targeted HCT116 sublines (Samuels et al., 2005) containing only the wild-type or mutant *PI3KCA* allele (Fig. 9A) were compared. Despite similar inhibition of Akt-mediated substrate phosphorylation (Fig. 9, B and C), the *PI3KCA* mutant cells were somewhat more sensitive to single-agent A-443654 (Fig. 9, D and E; 0 nM camptothecin), consistent with earlier reports using other Akt inhibitors (Samuels et al., 2005; She et al., 2008; Hanrahan et al., 2012; Slomovitz and Coleman, 2012). Particularly germane to the present question, A-443654 also sensitized the *PI3KCA* mutant subline to camptothecin to a much greater extent than the *PI3KCA* wild-type cells (Fig. 9, D–F).

Discussion

Results of the present study demonstrated that Akt inhibition synergistically enhanced the antiproliferative effects of melphalan and cisplatin. In contrast, the effects of combining A-443654 with topoisomerase poisons were much more dose- and context-dependent. Although low concentrations of A-443654 enhanced the effects of topoisomerase poisons, concentrations that inhibited Akt-mediated substrate phosphorylation by >80% blunted the antiproliferative effects of camptothecin and, to a more limited extent, etoposide in a variety of solid tumor cell lines, including those with activating *RAS* mutations, such as A549 and MDA-MB-231. The fact that similar observations were also obtained with MK-2206 and PDK1 siRNA rules out the possibility that this was unique to A-443654. In contrast, combinations of Akt inhibition and topoisomerase poisoning were synergistic in cells with activating *PI3KCA* mutations. These results have potential implications for ongoing development of PI3K/Akt pathway inhibitors.

Our previous results demonstrated that A-443654 enhances the cytotoxicity of tumor necrosis factor-related apoptosis

Fig. 7. Effect of PDK1 siRNA on BrdU incorporation into DNA and drug sensitivity. (A–C) Beginning 48 hours after the second transfection with PDK1 or control siRNA, A549 cells were harvested for immunoblotting with antibodies that recognize the indicated antigens (A) or pulsed for 30 minutes with 10 μ M BrdU, fixed, and stained with anti-BrdU antibody and PI as described in *Materials and Methods* (B). (C) Quantitation of the BrdU incorporation after siRNA PDK1 downregulation. Error bars represent the mean $\pm$ 1 S.D. from three independent experiments. (D–M) Beginning 48 hours after the second transfection with PDK1 siRNA (●) or, as a control, luciferase siRNA (○), A549 cells (D–J) or HeLa cells (K–M) were plated for 4 hours, exposed for 24 hours to the indicated concentrations of melphalan (D), cisplatin (E and F), camptothecin (G, H, and M), or etoposide (I–L), washed, and allowed to form colonies in drug-free medium. Error bars in D, E, G, I, and K represent the mean $\pm$ S.D. of triplicate aliquots in one of three independent experiments. Error bars in F, H, J, L, and M represent the mean $\pm$ S.D. of results from three (A549) or four (HeLa) independent experiments. CPT, camptothecin; DMSO, dimethylsulfoxide.

inducing ligand by inducing Mcl-1 downregulation (Kobayashi et al., 2005). In the present study, we extended these results by showing that A-443654 also enhances the effects of melphalan and cisplatin (Fig. 2). These results are consistent with

previous reports showing that inhibition of PI3K (Piccolo et al., 2004), overexpression of phosphatase and tensin homolog (Yan et al., 2006), or inhibition of Akt by different agents (Hideshima et al., 2006; Liu et al., 2007) also sensitizes cells to

Fig. 8. Effects of A-443654 on HCT116 cell substrate phosphorylation and clonogenic survival. (A) After HCT116 cells were treated with A-443654 for 24 hours, whole-cell lysates were subjected to immunoblotting with antibodies to the indicated antigens. (B) Phospho-GSK-3 β was quantitated as described in *Materials and Methods*. (C) HCT116 cells were treated with 0–75 nM A-443654 for 24 hours, washed, and allowed to form colonies. (D–L) HCT116 cells (D–F) or DLD1 cells (J–L) were treated for 24 hours with the indicated concentration of camptothecin (D–F and L) or etoposide (G–K) in the presence of diluent or A-443654 as indicated, washed, and incubated for 7 days in drug-free medium. CI values (F and I) were calculated from data in (D and G), respectively. Error bars in C, D, G, and J represent the mean $\pm$ S.D. of triplicate aliquots from one of three independent experiments. Error bars in B, E, H, K, and L represent the mean $\pm$ S.D. of three independent experiments. CPT, camptothecin; DMSO, dimethylsulfoxide.

Fig. 9. Effects of A-443654 on HCT116 cell clones with wild-type (wt) or mutant (mut) *PI3KCA*. (A) Sequencing of HCT116 cDNA showing sequence around nucleotide 3140 in the open reading frame of HCT116 sublines in which the mutant (top) or wild-type (bottom) *PI3KCA* allele was disrupted (Samuels et al., 2005) to yield cells with only wild-type or mutant allele, respectively. (B and C) After the indicated HCT116 clone was treated with A-443654 for 24 hours, whole-cell lysates were subjected to immunoblotting with antibodies to the indicated antigens. Blots were quantitated (C) as described in *Materials and Methods*. (D–F) HCT116 cells with wild-type (D) or mutant (E) *PI3KCA* were treated for 24 hours with the indicated concentration of camptothecin in the presence of diluent or increasing A-443654 concentrations as indicated. At the completion of the incubation, cells were washed and incubated for 7 days in drug-free medium. CI values (F) were calculated from data in D and E as well as additional results obtained using 50 nM A-443654. Error bars in D and E represent the mean $\pm$ S.D. of triplicate aliquots. Results are representative of three independent experiments.

cisplatin or melphalan. PDK1 siRNA likewise enhanced the effects of melphalan and cisplatin (Fig. 7, D–F), suggesting that the result is a bona fide effect of inhibiting the PI3K/Akt pathway.

Earlier studies reported that inhibition of the PI3K/Akt pathway also enhances camptothecin- (Saga et al., 2002; Knuefermann et al., 2003; Shi et al., 2005) or etoposide-induced death (O’Gorman et al., 2000; Knuefermann et al., 2003; Piccolo et al., 2004; Hodgkinson et al., 2006) in multiple cell types by diminishing Akt-induced survival signals. Consistent with these reports, we observed that low concentrations of A-443654 synergistically enhanced the antiproliferative effects of camptothecin or etoposide in several solid tumor cell lines, including A549, T98G, and MDA-MB-231, that lack *PI3KCA* activating mutations (Figs. 3 and 4). Interestingly, inhibition of Akt, as manifested by diminished phosphorylation of GSK-3, was incomplete at the A-443654 concentrations that contributed to this synergy (Fig. 1, B–G).

In view of the current practice of escalating drugs, including signal transduction inhibitors, to the maximum tolerated dose during clinical trials, we examined the effects of higher A-443654 concentrations in these *PI3KCA* wild-type cell lines. These higher concentrations, which induced more complete inhibition of Akt signaling (Fig. 1, B–G), diminished the impact of simultaneously adding topoisomerase I and topoisomerase II

poisons in a number of cell lines (Figs. 3 and 4). Although it is possible that these results reflect an effect of A-443654 on a currently unknown target, a possibility that must be considered with any small-molecule inhibitor or siRNA, several observations suggest that the antagonism is indeed mediated through Akt inhibition. First, two structurally and mechanistically distinct Akt inhibitors, the active site-directed inhibitor A-443654 (Luo et al., 2005; Shi et al., 2005) and the allosteric inhibitor MK-2206 (Hirai et al., 2010), exhibited similar effects (Fig. 3). Second, similar results were also observed with PDK1 siRNA in multiple cell lines (Fig. 7), suggesting that the effect is also a bona fide effect of inhibiting PI3K/Akt pathway signaling.

These antagonistic effects appear to be at odds with earlier reports that PI3K/Akt pathway inhibitors and topoisomerase I poisons synergize (Shi et al., 2005; Fala et al., 2008; Hirai et al., 2010). There are several potential explanations for these divergent results. First, we used inhibition of colony formation rather than induction of apoptosis as a readout of cellular effects. It is possible that this endpoint made it easier to observe the antagonism. Second, we used different cell lines from those used in earlier studies. Finally, because clinically achievable A-443654 concentrations are not known, we used A-443654 concentrations that inhibited Akt to varying extents. It is important to emphasize that the previously reported

synergy was observed in the present study, but only at concentrations that caused partial inhibition of Akt. When the A-443654 concentration was increased as little as 2-fold to achieve more complete Akt inhibition, the effects of adding camptothecin were diminished. These observations suggest a level of complexity that was not apparent from earlier studies.

Because the antagonism between PI3K inhibitors and topoisomerase poisons in *PI3KCA* wild-type cell lines was unexpected, a number of experiments were performed to shed light on the mechanism underlying this antagonism. Examination of camptothecin uptake by flow cytometry failed to show diminished drug uptake in the presence of A-443654 (Fig. 5). On the other hand, immunoblotting demonstrated diminished topoisomerase I and II expression (Fig. 1, B–D), providing one factor that might contribute to diminished sensitivity, although this effect was small at the 250–300 nM A-443654 concentrations that manifested antagonism. Moreover, A-443654 also diminished topoisomerase I expression in cells that displayed synergistic effects (Fig. 8A), suggesting that topoisomerase I downregulation is likely not a dominant determinant of antagonism observed in this study. In further experiments, A-443654 also inhibited BrdU incorporation into DNA (Fig. 6, A and B). Consistent with earlier observations that camptothecin and its analogs predominantly rely on ongoing replication to convert topoisomerase I–DNA cleavage complexes into cytotoxic lesions (Holm et al., 1989; D'Arpa et al., 1990), A-443654-induced inhibition of DNA synthesis was accompanied by decreased DNA damage (Fig. 6, C and D), suggesting that effects on DNA synthesis contribute to the antagonism observed between camptothecin and high A-443654 concentrations. To the extent that advancing replication forks convert topoisomerase II–DNA cleavage complexes into cytotoxic lesions (D'Arpa et al., 1990; Kaufmann, 1991), diminished DNA synthesis could contribute to the antagonism between etoposide and high A-443654 concentrations as well (Figs. 3 and 4). In view of the role of Akt in cell proliferation (see *Introduction*), this antagonism might complicate attempts to combine PI3K or Akt inhibitors with topoisomerase I poisons, particularly if PI3K/Akt pathway inhibitors are escalated to doses that give substantial inhibition of their intended targets.

A somewhat different picture emerged when cells with an activating *PI3KCA* mutation were examined. In the case of HCT116 cells, increased sensitivity to single-agent A-443654 was observed (cf. Fig. 8C versus Fig. 1, H–J). Moreover, Akt inhibition enhanced the cytotoxicity of camptothecin and etoposide (Fig. 8, D–I). Synergy was also observed when A-443654 was combined with etoposide or camptothecin in DLD1 cells, another line with a *PI3KCA* activating mutation (Fig. 8, J–L; data not shown). The enhancement of killing in HCT116 cells was greater when the mutant *PI3KCA* allele was present than when it was absent (Fig. 9). These observations suggest that *PI3KCA* mutation status might need to be taken into account not only when thinking of single-agent Akt inhibitor therapy, but also when considering combinations of Akt inhibitors with other agents.

In summary, the results presented demonstrate a previously unappreciated level of complexity when Akt inhibitors are combined with other anticancer agents. Whereas Akt inhibition or PDK1 siRNA enhances the antiproliferative effects of multiple DNA cross-linking agents, similar synergism is not uniformly observed with topoisomerase I and II

poisons. Instead, a high degree of PI3K/Akt pathway inhibition can antagonize the effects of topoisomerase I or II poisons, particularly in cells that lack activating *PI3KCA* mutations. When pilot studies of PI3K and Akt inhibitors combined with other agents are being planned, these observations might need to be considered.

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Authorship Contributions

Participated in research design: Gálvez-Peralta, Erlichman, Kaufmann.

Conducted experiments: Gálvez-Peralta, Flatten, Loegering, Peterson, Schneider, Kaufmann.

Contributed new reagents or analytic tools: Flatten.

Performed data analysis: Gálvez-Peralta, Flatten, Kaufmann.

Wrote or contributed to the writing of the manuscript: Gálvez-Peralta, Kaufmann.

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